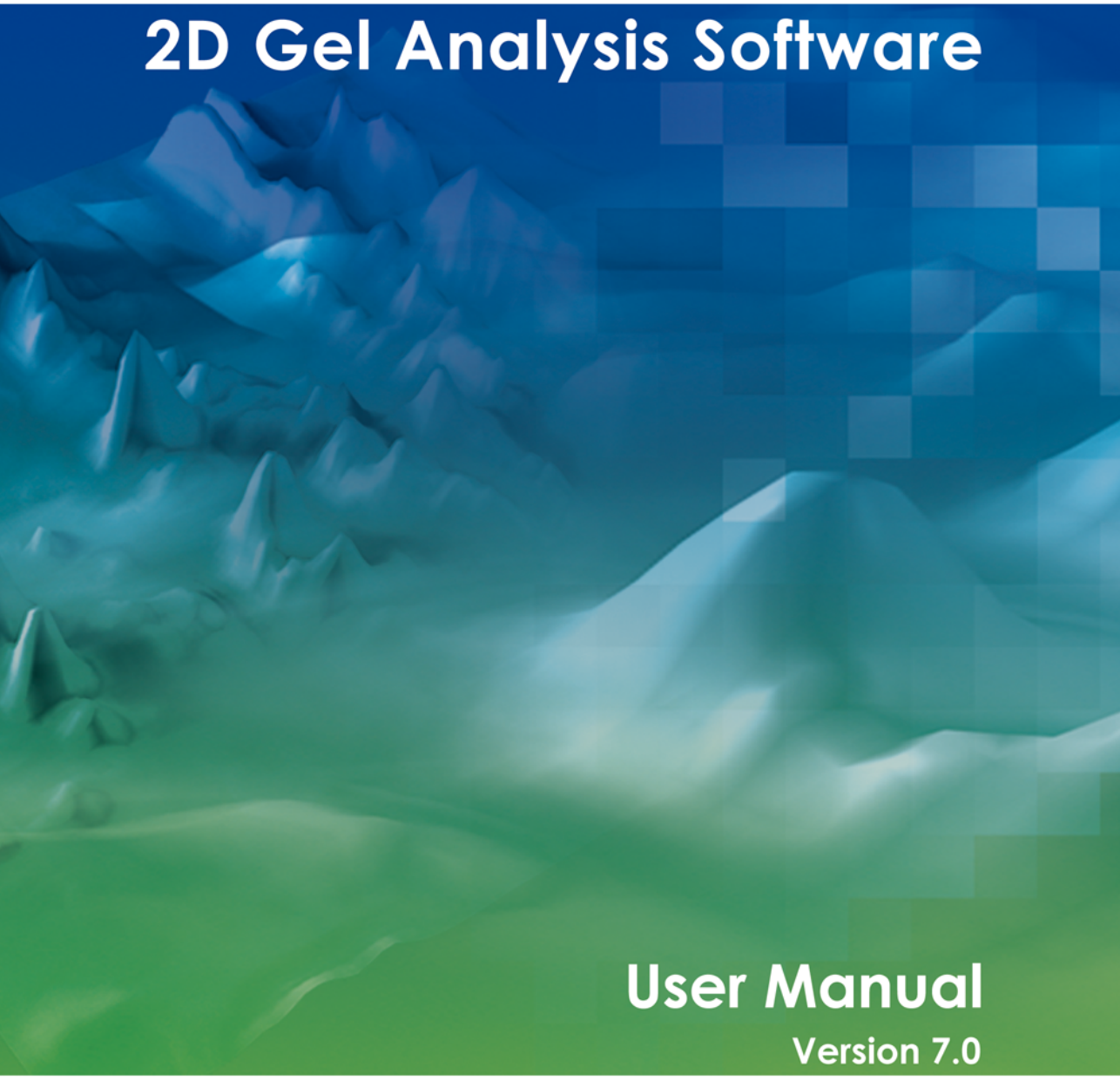
SIB melanie

2D Gel Analysis Software



User Manual

Version 7.0

SIB Swiss Institute of Bioinformatics

GENEBIO Geneva Bioinformatics SA

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1 Getting started

1.1 About the software

Melanie offers a unique and flexible solution for the comprehensive visualization, exploration and analysis of 2-D gel data.

This version of Melanie was developed by a team of top researchers from the Swiss Institute of Bioinformatics (SIB) in collaboration with Geneva Bioinformatics (GeneBio) SA and GE Healthcare. Professor Hochstrasser's group at the Geneva University Hospital contributed their real-world experience. The application has advanced to its current level primarily due to the input of users worldwide.

There are two modules of Melanie 7.0 available for purchase:

- **Melanie 7.0 DIGE**: To be used with conventional 2-DE and DIGE gels. It is fully functional, enabling you to add and import DIGE gels directly in the workspace. You can also co-detect DIGE gels using the algorithm created by the GE Healthcare DeCyder™ software development team as well as match, report, plot histograms and perform statistical analyses on DIGE gels.
- **Melanie 7.0**: To be used with conventional 2-DE gels. All menu commands related to DIGE are not functional and are grayed out in the graphical user interface.

1.2 System requirements

In order to install and run Melanie, your computer must satisfy the following requirements:

- Microsoft® Windows® XP, Vista, 7 or 8 operating systems.
- Administrative permission to install Melanie.
- At least 500 MB of RAM for Melanie (recommended: Intel dual-core processor with 1GB of RAM) and 768 MB RAM for Melanie DIGE (recommended: Intel dual-core processor with 2GB of RAM). The amount of memory required is determined by the number and size of image files to be processed simultaneously. Increased memory therefore enhances the performance when many and/or large images are analyzed.
- A high-quality display. To take full advantage of the software including the 3D View feature, the color resolution should be set to 24 bit (16.7 million colors). However, a color resolution of 8 bit (256 colors) is generally sufficient. It is recommended to use a screen resolution of at least 1024 x 768 pixels.

- Microsoft® Internet Explorer® 6 or Mozilla® Firefox® 3 or higher versions. A browser allows you to print reports and to access scientific databases on the Web.

1.3 Install the software

You can install Melanie from a CD-ROM or by downloading the installation package over the Internet. When the CD-ROM is inserted into the appropriate drive on your computer, the Setup Wizard starts automatically and gives a series of on-screen instructions. Alternatively, you can double-click on the icon of the installer file (.msi or .exe file) to launch the Setup Wizard.

The Melanie installer creates a default directory on your hard disk called Program Files\GeneBio\Melanie 7.0, in which the program files are placed. If you want to save the default directory in a different folder, then browse and select the location before continuing the installation.

Once installation is complete, it is recommended to restart your computer.

1.4 Licensing

Upon installation, Melanie is fully functional when analyzing the tutorial images, but it does not allow spot detection and matching on other gels. To analyze your own images, you must purchase and install an electronic license, or request a trial license. The availability of the license file is verified each time the software is launched.

There are two types of licenses:

- **Node-locked (Machine) license**: To be used on a single computer. It is practical when only a few computers are used for working with Melanie. A node-locked license file must be placed on the computer running Melanie.
- **Floating (Concurrent) license**: To be used by all computers networked to a a license server. It is useful when many users, but not all at the same time, need access to the software. The number of computers that can simultaneously work with Melanie depends on the license, and is administered via the license server. A floating license file must be placed on the computer running the license server. This server can either be installed on a computer running Melanie or on any other network computer (recommended: install on a network computer that is continually running).

Read on to learn how to obtain and place a license file.

1.4.1 Find the physical address of the computer

In order for GeneBio to create a license file, the physical address of your computer is needed. This address identifies the computer and is used by

the licensing system. If purchasing a node-locked license, send the physical address of the computer where Melanie is to be installed. If purchasing a floating license, send the physical address of the computer where the license server is to be installed.

To find the physical address while running the software:

1 Launch the software.

2 Choose ***Help > License Info***.

3 In the License Information window, click ***Copy to clipboard***.

4 Paste the clipboard content into an email and send this to melanie@genebio.com so that your license can be created.

To find the physical address of the license server, or when the software is not yet installed:

1 On the computer where the license file is to be placed (for example, the license server for a floating license), choose ***(All) Programs > Accessories > Command Prompt*** in the Windows Start menu.

2 In the Command Prompt window, enter ***ipconfig /all***. There must be a space between ipconfig and /all.

3 The response should contain several parameters for the network. Look for the line ***Ethernet adapter Local Area Connection***. Then look for the ***Physical Address***.

4 Note down the Physical Address among the displayed information, and send it to melanie@genebio.com so that your license can be created. The physical address must be correctly recorded for the license file to work.

1.4.2 Install the license server

This section only pertains to floating licenses. If you have a node-locked license, then skip to the next section.

To install the GeneBio ***license server:***

1 On the computer where the license server is to be installed, insert the Melanie CD-ROM.

2 Click ***Install*** GeneBio ***License Server***.

3 In the installation window, click ***Next***.

4 Accept the default installation path and start the installation. By changing the default installation path, the file paths in LMTOOLS will need to be updated accordingly.

5 Answer ***Yes*** to any question about Windows Firewall (this may or may not appear) in order for the license server to work properly.

6 LMTOOLS automatically opens once the installation is successfully completed.

7 Click the ***Config Services*** tab and ensure that all options are set as in Figure 1-1. The file paths shown (i.e., C:\Program Files\GeneBio\License Server\) are valid if the default installation was done.

8 Click ***Save Service***, even if no changes were made.

9 Leave LMTOOLS open and proceed to the next section.

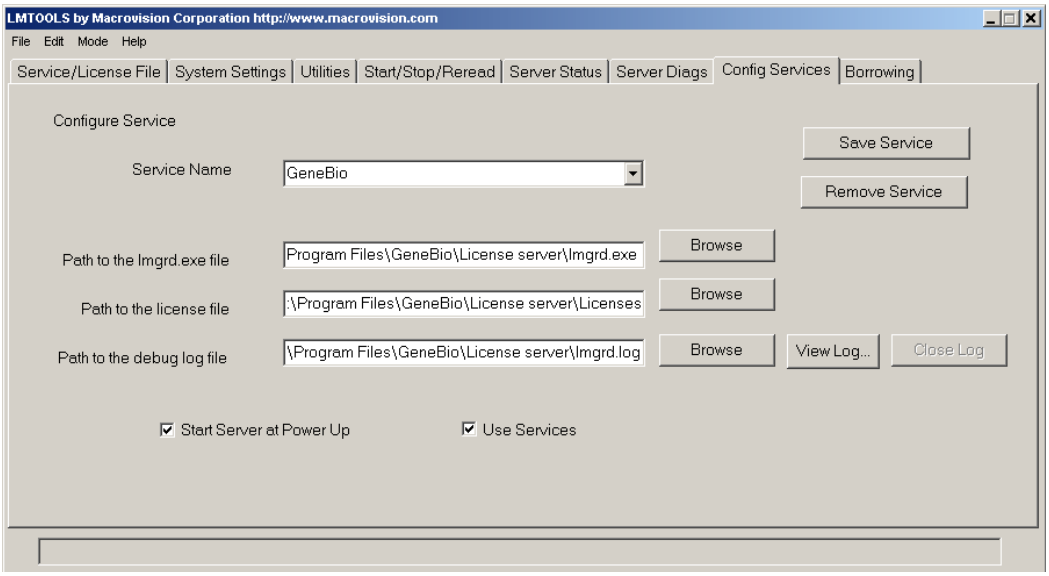


Figure 1-1. The Config Services tab in LMTOOLS.

1.4.3 Place a license file

You will receive the license file (with a .lic extension) from GeneBio once you have sent the physical address of your computer.

- For a node-locked license, the file must be placed in the Melanie installation folder (C:\Program Files\GeneBio\Melanie 7.0 by default).
- For a floating license, the file must be placed in the Licenses subfolder of the License server installation folder (C:\Program Files\GeneBio\License server\Licenses by default).

1.4.4 Start the license server

This section only pertains to floating licenses. If you have a node-locked license, then skip to the next section.

To start the GeneBio ***license server:***

1 Select the ***Start/Stop/Reread*** tab in LMTOOLS.

2 Click ***Start Server*** (Figure 1-2).

3 Close LMTOOLS.

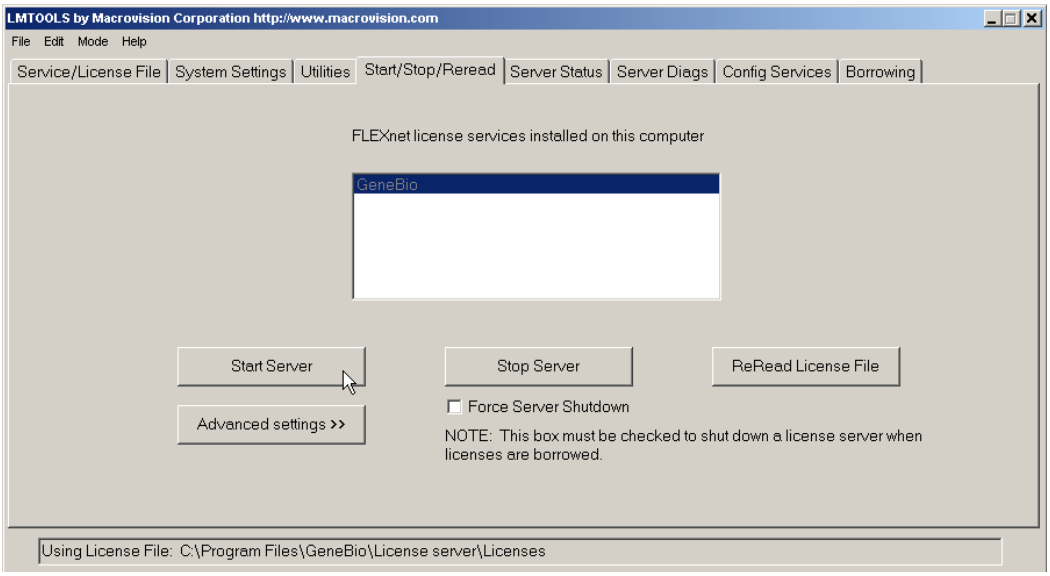


Figure 1-2. Starting the server from the Start/Stop/Reread tab in LMTOOLS.

1.4.5 Test the license file

In order to test that the license file is correctly placed, start Melanie on all computers where the software is installed.

For a floating license, a FLEXnet™ License Finder window displays asking to specify the License Server System or License File. Choose the first option and enter the name of the computer where the license server is installed. Click ***OK***.

1.5 Launch the software

Double-click the Melanie icon (Figure 1-3) found on your computer's desktop to start the software. Alternatively, go to ***(All) Programs > Melanie 7.0*** in the Windows Start menu. A splash page appears while the software is loading. It will disappear automatically.

Figure 1-3. Melanie icon.

1.6 Resources

1.6.1 Help

Tutorials

Two tutorials are provided with Melanie. The ***Non-DIGE Tutorial*** and ***DIGE Tutorial*** documents are available from the ***Help*** menu in the software, from the ***Windows Start*** menu, and from the folder Doc in the software installation directory. The corresponding image files can be found in the folder Tutorials/Images in the software installation directory.

Non-DIGE Tutorial

The 12 sample gels used in this tutorial come from an experiment that aims to study protein expression changes between four conditions. Cells were grown on two different substrates (substrate A and substrate B) and underwent one of two treatments (treatment 1 and treatment 2). Three biological replicates have been run for each condition.

Condition	Substrate	Treatment
A_T1	Substrate A	Treatment 1
A_T2	Substrate A	Treatment 2
B_T1	Substrate B	Treatment 1
B_T2	Substrate B	Treatment 2

DIGE Tutorial

The sample gels used in this tutorial come from an experiment that aims to study protein expression changes between control and treated groups of bacterial cultures.

In this study, eight sample lysates were prepared; four were derived from treated bacterial cultures and four were derived from un-treated (control) bacterial cultures. Aliquots from each sample were taken and pooled to prepare a standard sample (pooled internal standard). The samples were run on four gels. The control and treated samples were alternatively labeled with Cy3 or Cy5 (dye swap) to avoid dye-bias. The standard was labeled with Cy2 and run on each gel in the experiment.

Gel	Cy2	Cy3	Cy5
Gel 01	Pooled internal standard	Control A	Treated A
Gel 02	Pooled internal standard	Treated B	Control B
Gel 03	Pooled internal standard	Control C	Treated C
Gel 04	Pooled internal standard	Treated D	Control D

A preparative gel, including reference markers, was run on the pooled sample and post-stained with a Fluor total stain to pick spots and identify them by mass spectrometry (MS).

Tutorial projects

When the software is started for the very first time, two tutorial projects Tutorial and TutorialDIGE are restored. Each project contains the three first images of the corresponding experiment (described above). The

project can be completed with the remaining images that can be found in the folder Tutorials\Images in the Melanie installation directory. In this way, you first get a view of what the project structure looks like, and can then learn to add images to a project.

Note: *If* Melanie *was already used on your computer before, you can rename the file Experiment.mws in Documents and Settings\[username]\My Documents*Melanie*\Projects to make sure the tutorial projects are restored.*

User manual

Melanie is intended to be intuitive and comprehensive. In order to exploit the software's full potential, refer to the User Manual. You will find detailed explanations of all the features and functionalities. The chapters in this manual are generally organized according to the logical sequence of a 2-DE gel analysis, although expert users will agree that some steps can be inverted or repeated at some point.

1.6.2 Customer support

GeneBio provides technical and scientific support for Melanie. Please contact us if any problems arise during the installation or use of the software. Our support team is happy to help you.

How to contact us

By email: melanie-support@genebio.com.

By phone: +41 22 379 50 50.

By fax: +41 22 379 58 58.

Product, system and license information

Launch the software and choose ***Help > About Melanie*** to obtain product, system and license information. The product name, version number and version date that are displayed will be requested in technical support issues. You can view system and license information by clicking on the corresponding buttons.

The ***Copy to clipboard*** button in the License Information window can be used to paste license data into an email. In the System Information window, find facts about your computer or choose ***File > Export*** to save all the information in a text file.

We listen

The GeneBio team is attentive to your suggestions. Many of the enhancements to the software are a direct result of conversations with our customers. We truly appreciate any comments, criticisms or ideas that would help us to improve the software. Please do not hesitate to contact us by email at melanie-support@genebio.com.

1.7 What's new?

The graphical interface and user interaction modes have been entirely revamped. The streamlined analysis in Melanie now offers enhanced usability and speed.

Features have been redesigned to help minimize manual spot editing and repetitive match editing. Ultimately, tasks are simplified and the reliability of the results are increased.

The most important new features in Melanie, version 7.0 are listed below:

- Fully dynamic tables, histograms, plots and 3-D views in which both content and selection are continuously updated to stay synchronized with the corresponding sheet that contains the gel images.
- Simplified import and visualization of images
- Improved population matching
- Management of multiple matches and composite spots
- Reorganized menu structure with icons
- Custom and context-related toolbars
- One-click to choose desired layout of sheets and panes
- Option to surround spot selections by boxes, for easier identification
- Single tool to select/edit spots and annotations
- Dedicated landmark tool
- Measure tool to compute pixel, pI, MW, or real-world (centimeter or inch) distances between spots
- Reviewed contrast adjustment feature
- New 3D View
- Customized report templates
- Adaptive display of histograms
- And much more...

2 Graphical user interface

2.1 About the interface

The graphical user interface (GUI) is divided into four main parts, shown in Figure 2-1. They are the Menu Bar, the Toolbars, the Status Bar, and the Display Zone.

The Display Zone is the center of the interface. This is where gel images are arranged in sheets and panes. The Workspace and any reports are dockable windows found along the edges of the Display Zone.

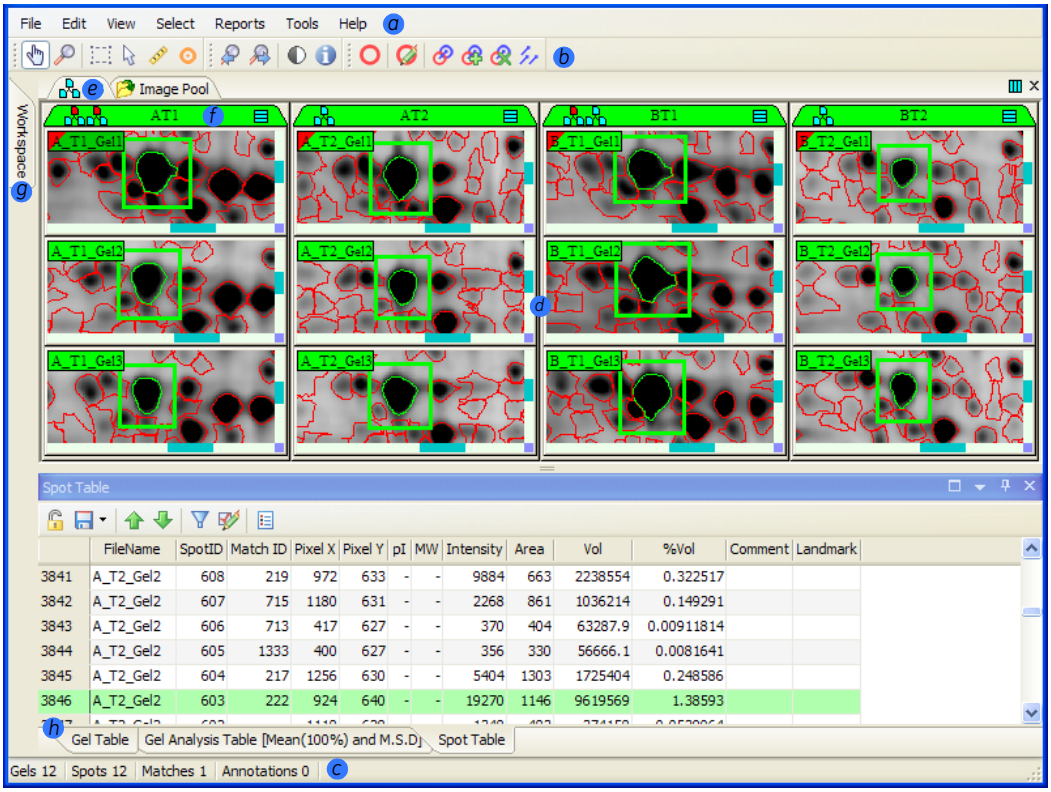


Figure 2-1. The Melanie window. (a) Menu Bar, (b) Toolbar, (c) Status Bar, (d) Display Zone, (e) Sheet, (f) Pane, (g) Workspace, and (h) Reports.

2.2 Menu bar

You can choose actions to be performed during your analysis from the Menu Bar. The menus are context related. This means some of the commands may not be available all of the time and either go away or

are grayed out. For example, the Select menu allows you to select spots, matches and annotations for detection and matching. Therefore, this menu is neither available nor displayed while viewing and editing images in the Image Pool.

Menu	Description
File	Close, save, import, export, print and other basic operations. You can also exit Melanie.
Edit	Undo/redo the last operations, show a history of operations, or edit (add, modify, delete) specific gels, spots, annotations or matches. You can also edit spot sets and enable spots.
View	Modify the settings for grid lines, profile or overview in the display, align images, show dual color or spot overlap in the current sheet, or change the way gels, spots, annotations or matches are visualized.
Select	Select specific spots, spot sets, annotations or matches.
Reports	Display tabular or graphical information on about selected gels, spots, annotations or matches, and compute differences and similarities between gel images. The data analys is based on robust statistics, factor analysis, and statistical tests.
Tools	Change display, quantification, and other options at the software level and customize the user interface (custom toolbars, keyboard shortcuts). Create and control a calibration tablet while working in the Image Pool .
Help	Access documentation and obtain license, product, version, or system information.

Most menu commands also have toolbar icons and/or keyboard shortcuts.

2.2.1 Keyboard shortcuts

Keyboard shortcuts, when available, are designated on the right-hand side of the corresponding menu command. A list of all shortcuts is given in the Appendix. Please note the logic behind the key combinations:

Ctrl is used to maneuver gels.

Shift is used to maneuver spots.

Alt is used to maneuver annotations.

Ctrl + Shift is used to maneuver matches.

You can create your own keyboard shortcuts for menu commands.

> **CAUTION!** A new keyboard shortcut must be unique. Be careful not to duplicate a keyboard shortcut that is already assigned to another menu command. You risk destroying an existing shortcut. However, a given command can have several, different keyboard shortcuts.

To add your own keyboard shortcut:

1 Choose ***Tools > Customize***.

2 Click the ***Keyboard*** tab.

3 Select the menu in the ***Category*** drop-down list and then select the command for which you want to create a shortcut (Figure 2-2).

4 Check the ***Key assignments*** list to see if the shortcut is currently assigned.

5 Click in the ***Press new shortcut key*** box and then press the new combination on your keyboard.

6 Click ***Assign***. The software warns you when the shortcut is already assigned to another command and asks if you want to re-assign it. Click ***No*** and come up with a different shortcut.

7 Click ***Close***.

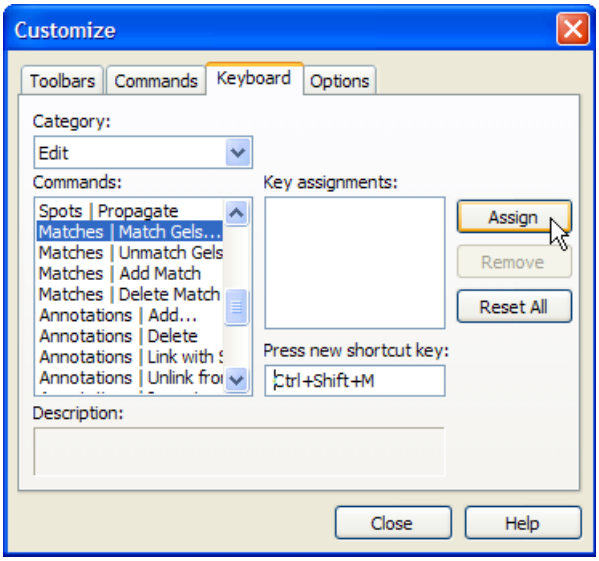


Figure 2-2. Defining keyboard shortcuts in the Customize window.

2.3 Toolbars

2.3.1 Default toolbars

The standard toolbars provided are designed so that you can quickly manipulate your gel data and apply the most frequently used features. Each of the tools are described in detail elsewhere in the User Manual.

Tools

This toolbar contains the basic tools of the software. More importantly, their functions (Move, Zoom, Region, Selection, Measure and Landmark) are not available in any menu. Although they do have corresponding keyboard shortcuts.

Display

This toolbar contains the options Undo Zoom/Move, Redo Zoom/Move, Adjust Contrast and Cursor Information Window.

Image

The image toolbar (Rotate, Flip, Invert Gray Levels, Crop and Add Files to Project) is contextual; it is only available when working with images in the Image Pool.

Detect And Match Spots

This toolbar is only displayed when working with gels in a Match or Class sheet (i.e., once gels have been imported into a project). You can Detect, Enable Edit, Match Gels, Add Match, Delete Match and Show Vectors.

Edit Spots

This toolbar is only available when spot edition is enabled. Choose ***Edit > Spots > Edit Enabled*** to get into this mode.

2.3.2 Customize toolbars

Toolbars can be configured according to your individual specifications.

Toolbar position

To change the position of a toolbar, click the left edge and drag the toolbar to the position you want. You can drag a toolbar to any of the edges of the GUI. When a toolbar is dragged outside of the frame of the Melanie window, it becomes a floating window.

Toolbar format

By default, the toolbar icons in the software are small (16x16 pixels). You can choose to display large icons (24x24 pixels) for better visibility. In addition, when you move the mouse over an icon, a screentip appears. Perhaps you prefer to hide all the screentips, or just remove the keyboard shortcuts for the tools from the screentips.

To modify the toolbar format:

1 Choose ***Tools > Customize***.

2 Click the ***Options*** tab in the Customize window.

3 Select the desired options in the ***Other*** section.

4 Click ***Close***.

Custom toolbars

You can create your own toolbars with icons for the functions you use most commonly.

To create a custom toolbar:

1 Choose ***Tools > Customize***.

2 Click the ***Toolbars*** tab in the Customize window.

3 Click ***New*** to create a toolbar. Enter a name for the toolbar and click ***OK***. An empty toolbar is created below the existing ones.

4 Click the ***Commands*** tab in the Customize window.

5 Select the menu from the ***Category*** list. Then select the command for which you want to create an icon and drag it to the empty toolbar.

6 Repeat step 5 to add icons to the toolbar.

7 Click ***Close*** in the Customize window.

2.4 Status bar

The Status Bar at the bottom of the Display Zone is an important resource. It indicates the total number of gels, spots, matches and annotations that are selected in the current sheet.

If you move your mouse over a gel image, the Status Bar also indicates the X and Y coordinates at the cursor position, as well as the image intensity. The unit of the coordinates can be changed in ***Tools > Options***, under the ***Display*** tab.

2.5 Display zone

Gel images are opened in the Workspace and viewed in sheets and panes in the Display Zone (Figure 2-3). Their layouts can be arranged according to your requirements.

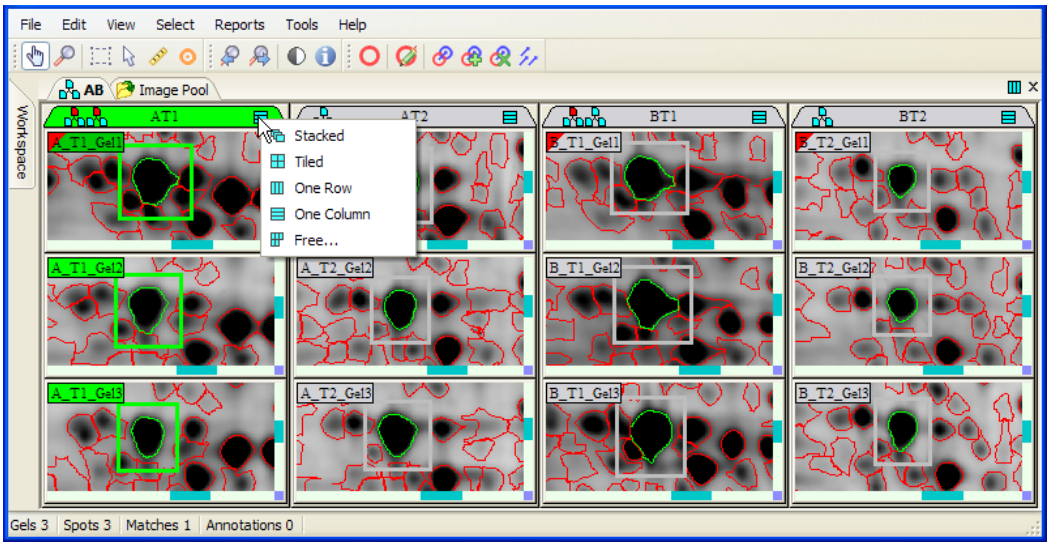


Figure 2-3. The Display Zone. There are two sheets open here, including the Image Pool. The current sheet AB is in front and contains four panes (AT1, AT2, BT1 and BT2). Only the pane AT1 is selected (green tab). By clicking the Layout icon for the pane, different options (Stacked, Tiled, One Row, One Column, Free) are displayed. Similarly, click the Layout icon in the upper right corner to re-arrange a sheet.

2.5.1 Sheets

When no dockable windows (Workspace, reports) are open, sheets occupy the entire Display Zone. Each sheet has a tab, with its name and an icon representing its type:

- **Image Pool**: This sheet contains images for viewing and basic processing.
- **MatchSet**: This sheet is opened by right-clicking on the name of a match set in the Workspace. Spot detection and matching must be carried out on this type of sheet.
- **Class**: This sheet is opened by right-clicking on the name of one or several classes in the Workspace. To carry out advanced expression analysis, you must work in this type of sheet.

When you move the mouse cursor over a sheet tab, the screentip specifies the type.

Selection
The current sheet is always in front and its name is in bold. Select a sheet by clicking on its tab.

Close
Click the ***Close*** icon in the upper right corner of the current sheet when you are finished working with it.

Layout
The ***Layout*** icon to the left of the ***Close*** icon can be used to choose the arrangement of the panes in a sheet.

Option	Description
Stacked	Panes in a sheet are one on top of another.
Tiled	Panes in a sheet are side by side.
One Row	Panes in a sheet are in a single horizontal line.
One Column	Panes in a sheet are in a single vertical line.
Free	You specify the number of panes laid out horizontally and vertically in a sheet.

2.5.2 Panes

There can be one or more panes in a sheet (see Figure 2-3). Each pane has a tab with its name. On the left side of the tab, one or more icons describe the match or class hierarchy.

Selection
Select a pane by clicking on its tab. Use the Shift or Ctrl keys to make multiple selections. Click the sheet tab to select all panes in a sheet. Selected panes have green tabs.

By default, panes are laid out in Tiled mode. When working in a different mode like Stacked, bring a hidden pane to the front by clicking on its tab.

Layout
Click the ***Layout*** icon on the right side of the tab to change the arrangement of the images in a pane. The options are the same as for panes in a sheet.

2.5.3 Images

By default, the gel name is displayed in the upper left corner of an image. The color of the name indicates whether the image is selected (green) or not (gray). If an image name has a red corner, this means that it is the reference image for the matching. If an image name is a darker green or darker gray than the other images, then it is the current

sheet reference. All other images in the sheet are compared to this sheet reference when using the menu commands in ***View > Sheet***.

To hide the image names, choose ***View > Global > Show Gel Names***. The image name is replaced by three dots.

Move the mouse over an image name to view a screentip specifying the match hierarchy. In a Class sheet, the class and path to which it belongs are also given. If the image is the sheet reference, then this fact is included in the screentip.

Selection
Select an image by clicking on its name. Use the Shift or Ctrl keys to make multiple selections. Click the pane tab to select all images in a pane.

When images are hidden like in Stacked mode, bring an image to the front by clicking on its tab at the bottom of the pane. Quickly sift through images using the Page Up and Page Down keys on your keyboard or click on the navigation triangles in the lower right corner of a pane.

Scrollbars
The scrollbars on the right and bottom edges of each image can be used to change the zoom factor of an image by dragging one of the ends of the scrollbar. You can also move the visible area of the gel horizontally or vertically by clicking in the middle of the scrollbar and dragging left/right or up/down.

Click on the gray square at the intersection of two scrollbars to reset the image to its full image size.

If you want to view a specific area of your gel image, choose ***View > Global > Scrollbars > Adjust***. In the Adjust Visible Area window, set the exact horizontal and vertical start and end coordinates for the area to be displayed. You can do this in terms of different units: Pixel coordinate, Percentage and Real coordinate (pI and MW). Please note that pI_MW annotations must be defined in order to use this function.

To hide the scrollbars, choose ***View > Global > Scrollbars > Show***. In that case, You can still move and zoom images by using the Move and Zoom tools in the toolbar.

2.5.4 Switch order

You can change the order in which images are displayed by dragging the gel name onto another image. It is then inserted before this image. Similarly, you can re-order panes by dragging their tabs to a new position.

Swap panes or images by choosing ***View > Sheet > (Navigation >) Switch*** or the Ctrl+F shortcut. This reverses the last re-ordering operation applied to a pane or image.

2.6 Workspace

The Workspace plays an important role in the software. It allows you to organize your gels into projects, to specify how the gels are to be matched together, and to define your classes (or groups) for statistical analysis. A brief description of the Workspace window is presented here. The utility of the Workspace is discussed in another chapter.

The Workspace window is a dockable window and has two main parts: the Workspace toolbar and the Navigator (Figure 2-4).

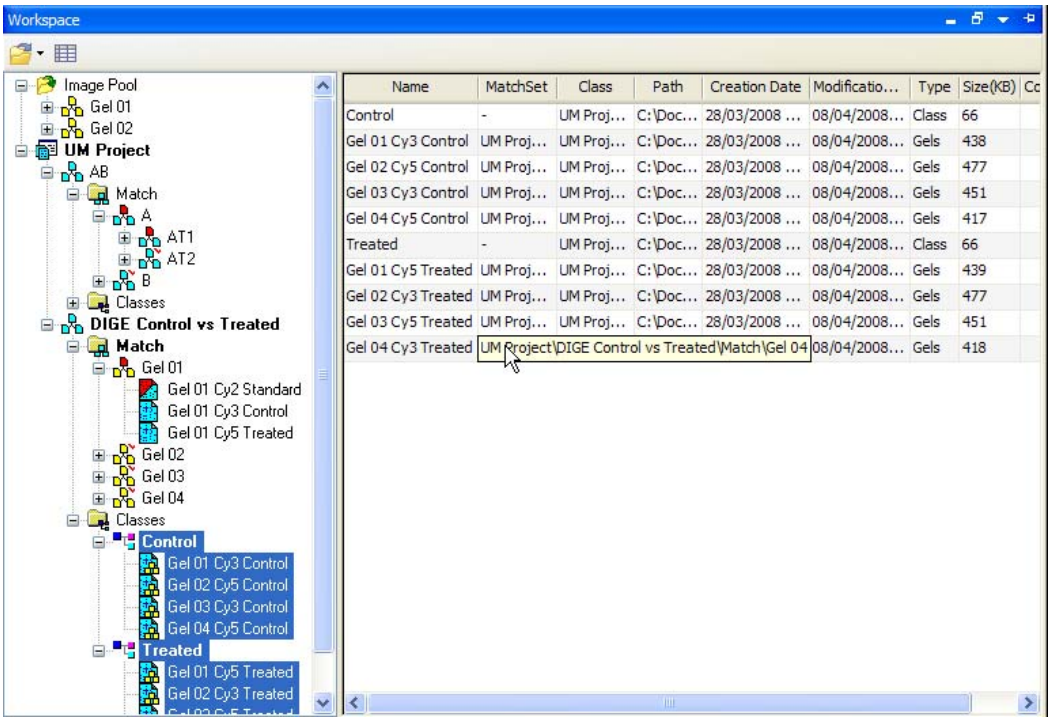

Workspace
Image Pool
Gel 01
Gel 02
UM Project
AB
Match
A
AT1
AT2
B
Classes
DIGE Control vs Treated
Match
Gel 01
Gel 01 Cy2 Standard
Gel 01 Cy3 Control
Gel 01 Cy5 Treated
Gel 02
Gel 03
Gel 04
Classes
Control
Gel 01 Cy3 Control
Gel 02 Cy5 Control
Gel 03 Cy3 Control
Gel 04 Cy5 Control
Treated
Gel 01 Cy5 Treated
Gel 02 Cy3 Treated
Name
MatchSet
Class
Path
Creation Date
Modificatio...
Type
Size(KB)
Control - UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Class 66
Gel 01 Cy3 Control UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 438
Gel 02 Cy5 Control UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 477
Gel 03 Cy3 Control UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 451
Gel 04 Cy5 Control UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 417
Treated - UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Class 66
Gel 01 Cy5 Treated UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 439
Gel 02 Cy3 Treated UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 477
Gel 03 Cy5 Treated UM Proj... UM Proj... C:\Doc... 28/03/2008 ... 08/04/2008... Gels 451
Gel 04 Cy3 Treated UM Project\DIGE Control vs Treated\Match\Gel 04 08/04/2008... Gels 418

Figure 2-4. The expanded view of the Workspace window. The Workspace toolbar and Navigator (at the left) are always visible. The file details in the expanded view (at the right) are only displayed when desired.

2.6.1 Toolbar

Commands to create new projects, add files to projects, remove, backup and restore projects are found under the ***Project*** icon in the Workspace toolbar. .

Click the ***Expanded View*** icon to enlarge the Workspace window to include details of all files selected in the Navigator. The files can be sorted in ascending or descending order by clicking on a column header. Click again on the ***Expanded View*** icon to hide the file information.

2.6.2 Navigator

The Navigator displays all files and folders you can view (Image Pool) and analyze (projects). The look and feel is similar to Windows Explorer. There is a hierarchical structure of folders, subfolders and files that can be expanded or collapsed, dragged and dropped in a new location, copied and pasted, etc.

When launching the software for the first time, there is an empty Image Pool folder. Images are opened through the Image Pool. To further analyze images (detect, match, carry out statistical analysis, etc.), they must be transferred to a project. You can work on many projects in the Workspace, each of which will contain one or more root structures with Match and Classes subfolders.

Current sheet
Any item in bold in the Navigator corresponds to items displayed in the current sheet.

Contextual menus
A contextual menu containing relevant options appears when you right-click an item in the Navigator.

Navigator icons
Each folder type in the Navigator has a specific icon (Image Pool, Project, Match, Class). In addition, images and match set folders have icons that indicate their status. You can distinguish DIGE gels from non-DIGE gels, know which gels have been detected and matched, and recgonize gels used as references for matching.

Icon	Meaning
	Undetected image / reference image.
	Detected image / reference image.
	Matched gel / reference gel.

Table 2-1. The Navigator icons inform you about the type and status of the image.

2.7 Reports

Reports are highly practical for organizing and describing your gel data. They make it much easier to process all of the information.

Reports are not necessarily in table format. Graphical representations of data such as histograms, scatter plots and 3D views are treated as reports as well. All reports are dockable windows.

Report	Description
3D View	A three-dimensional view of selected gel regions or areas around selected spots.
Analyze Gels	Information about each selected spot match such as its Match ID, value for each spot in the match, and chosen statistical measures calculated on all spots in the match. Scatter Plots also provide information (slope, offset, correlation coefficient, fitting error) that compare the spot values for two gels. The Match Statistics Table displays the number of matches and percentage of matches for each gel at the selected level of the match hierarchy. The DIGE Histogram displays the frequency distribution of the DIGE volume ratios.
Analyze Classes	Central tendency, dispersion, and overlapping measures for classes of gels computed for all selected matches. Differences between the spot values in several classes can also be quantified with Statistical Tests such as ANOVA, Mann-Whitney U test and Kolmogorov-Smirnov test.
Gel Table	Summarized information about the selected gels, such as Gel ID, file name and path, gel calibration data, gel resolution and size, number of detected spots, user defined properties such as sample type, staining or date of the experiment, and much more. Predefined templates (Properties, Files, Descriptions, Calibration) allow you to quickly display a specific subset of fields. You can also create custom templates.
Spot Table	Specific information about selected spots such as Spot ID and coordinates, quantification values, attached labels, etc.
Annotation Table	Information about annotations, including the label content for each category, the annotation coordinates and Spot ID (if the annotation is linked to a spot).

2.7.1 Dynamic content

Report content is continuously updated. A report selection is synchronized to reflect the most current data from the corresponding sheet that contains the gel images.

Notice that reports are attached to their corresponding sheet. If the sheet is closed, the reports will be closed as well.

2.7.2 Content based on enabled spots

By default, all spots are enabled and therefore represented in the reports. But the content of reports can be limited to a subset of spots by disabling spots that are not of interest. See 6.4.3 to learn more about enabling/disabling spots.

2.7.3 Toolbars

Most reports have a toolbar with the following icons in addition to some report-specific icons and functionalities that are described in later chapters of the User Manual:

Suspend synchronization

By default, the selection in the report is continuously synchronized with the corresponding sheet that contains the images and with other reports. Click the ***Suspend Synchronization*** icon to stop the synchronization and render the selection in the report independent of the selection on the gel, and vice versa.

Save

Enter a name and select the desired format for the file to be saved. Tables can be saved in tab-delimited text format (.txt), as a Microsoft Excel Workbook (.xls), or in XML format (.xml). Graphics can be saved in PNG, TIFF™ or BMP formats.

Print

Normal print options are available when printing graphical reports. When printing tabular reports, the table is first displayed in your default Web browser. This is because the XSL stylesheet located in the Template\Reports folder of the Melanie installation directory is used to transform the XML report into an attractive table. You can then use the print option in your browser to get a printout.

Copy to clipboard

Export your data directly into another application. First select the desired lines in a table, or the desired graphics in a window, using the Shift or Ctrl keys. Then copy the selection to the clipboard by choosing the ***Copy to Clipboard*** icon. Paste directly into the preferred software.

Previous selection

Click the ***Previous Selection*** icon to skip to the first selected item encountered when scrolling towards the top of your table. When only one row is selected, this selects the previous row in the table.

Next selection

Click the ***Next Selection*** icon to skip to the first selected item encountered when scrolling towards the bottom of your table. When only one row is selected, this selects the next row in the table.

Settings

Click this icon to change the display settings for your report. In most cases, this means setting the visibility (hidden or shown) of the columns. In some cases (Histograms and Match Statistics Table), you can define other display options.

Select by value

This feature allows you to select items in the report based on a numerical search criterion. Click the Select by Value icon, then select the measure (i.e., column) you want to use for refinement, and finally set the lower and/or upper limits of your search interval.

2.7.4 Customize reports

Sorting data in columns

Data in tabular reports can be sorted by the column content. If you click once on the header of a specific column, a triangle is displayed indicating that the column's numerical or textual data is sorted in ascending order. When you click once more on the header, the triangle inverts indicating that the data is sorted in the opposite order. Note that ascending order means that numbers are sorted from 0 to 9 and text is sorted from A to Z.

Column visibility

Load a predefined template or save your own template indicating what columns should be hidden or shown using the ***Settings*** icon in a report toolbar. This is particularly useful when you only want the essential information to appear for clarity or for printing purposes.

To apply an existing report template:

1 Click the ***Settings*** icon in a report toolbar.

2 In the template window (Figure 2-5), select a template name with the ***Load*** icon. By default, only the Gel Table has more than one predefined template (Properties, Files, Decriptions, Calibration).

3 Click ***OK***.

To create and save your own report template:

1 Click the ***Settings*** icon in a report toolbar.

2 In the template window, select (box is checked) the attributes that you want to show in the report and deselect (box is unchecked) the attributes to hide columns.

3 To save the template for later use, click the ***Save*** icon. Choose a name from the list or select ***<New...>*** to create one. Click ***OK***.

4 Click ***OK*** in the template window.

To delete an existing report template:

1 Click the ***Settings*** icon in a report toolbar.

2 In the template window, click the ***Remove*** icon and choose the template to be deleted. Click ***OK***.

3 Click ***Yes*** when asked for confirmation.

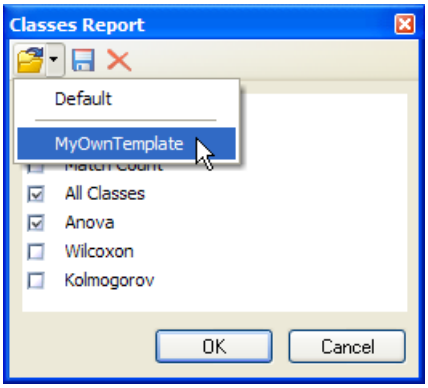


Figure 2-5. Report template window.

Column order
You can reorganize table columns directly in a report window. To do so, drag the column header to its new position. You will see red arrows at the insertion point.

Column size
You can enlarge or reduce the column size. Drag the right edge of the column header until the column is the width you want. To resize columns so that their whole content is displayed, double-click on their separator. The column to the left of the cursor is resized.

2.7.5 Edit table cells

In the Spot table, cells containing annotation labels are editable. Double-click in a cell to start typing or editing your label. When finished, a single click in any cell quits the editing mode.

Please note that annotation categories of the data type Set are displayed as check boxes. A checked box means that the item belongs to the Set. An empty box means that it does not belong to the Set.

The same is true for spot sets represented in any of the tables: a checked box means that the spot belongs to the set. An empty box means that the spot does not belong to the set.

To check several boxes (i.e., lines) simultaneously:

1 Use the Shift or Ctrl keys to select the rows in which you want to check or uncheck the box.

2 Click in the box of one of the selected rows. All rows are now either checked or unchecked.

2.8 Dockable windows

The Workspace, reports, and Adjust Contrast window are dockable windows. Dockable windows make it easier for you to work with numerous windows at the same time. These windows can be docked (i.e., fixed in place) against the left, right, top or bottom edges of the Melanie window and always lie on top when visible. A visible window can be in one of three modes:

Pinned

The pinned mode enables dockable windows to be locked into position around the edges of the Melanie window. Once a window is pinned, you can move it to a different location by dragging the title bar (Figure 2-6). Guides indicate where the window may be docked. By moving the cursor over the guides, a shaded blue box appears showing where the window will reside if the left mouse button is released. If you move the dockable window to a non-predefined location, it becomes a floating window. Moving a docked window may affect the location and size of other docked windows.

Un-pinned

A visible window in un-pinned mode automatically collapses when not in use to become a tab at the edge of the Melanie window (in Figure 1-7, the Workspace is in this mode). When you click on a docked tab, the window slides back into view and is ready for use. You can also click on Minimize to minimize a window in un-pinned mode.

Floating

A dockable window in floating mode will always appear on top. It can be dragged to any position within the software or even outside the Melanie window. You can switch in and out of floating mode by double-clicking on the title bar of a dockable window.

Tabbed groups

Dockable windows can be organized into tabbed groups. This feature extends your ability to maximize the use of limited screen space by combining multiple dockable windows into one window. In order to form a tabbed group, drag the title bar of a dockable window into the center of another. You will see the nested tabs at the bottom of the docked window. In order to separate a tabbed group, drag a tab away from the docked window or double-click on the tab. Please note

that tabular reports and graphical reports cannot be grouped together.

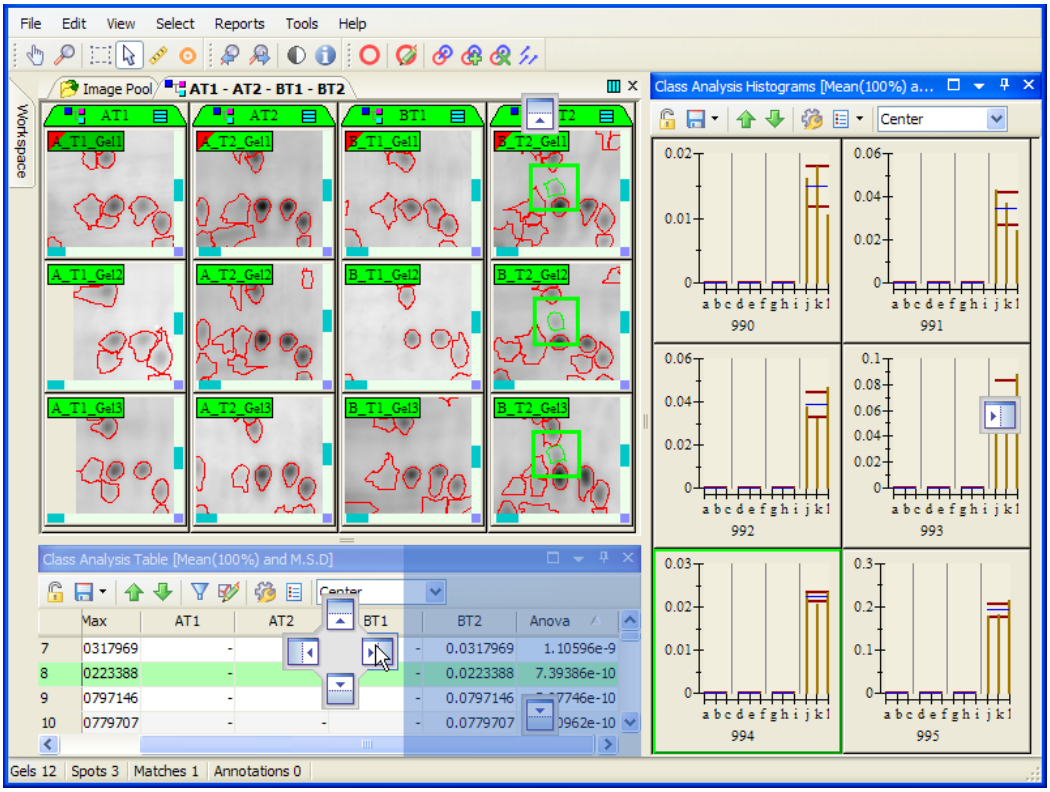


Figure 2-6. The Class Analysis Histograms window previously docked at the right edge will now be docked to the right of the Class Analysis Table window, in the lower left corner of the Melanie window.

2.9 Options

You can set various parameters that influence your work in Melanie. These settings are accessed by choosing ***Tools > Options*** in the menu. More detailed information about the options are provided in the related chapters of the User manual. However, an overview of the settings, per tab, is given below.

Display

- Indicate the default spot colors (for enabled, disabled, selected and overlapped spots) and the color for the match vectors.
- Choose the units to express coordinates in the Status Bar and Measure tool.

- Indicate whether spot selections should be surrounded by boxes, for easier localization.

Annotation

- Define the annotation categories that should always be available in the software, and set their attributes and display properties.

Gel descriptions

- Define the gel descriptions that should always be available in the software.

Quantification

- Specify the spot quantification value to be used for non-DIGE (Value) and DIGE (DIGE Value) experiments. This spot quantification value is used in Gel and Class Analysis Reports.
- Compute the spot areas in mm^2, based on gel resolution (default), or pixels.

General

- Specify whether the raw image data of newly opened files should be kept in memory.

  With the option ***Keep image in memory*** selected, the image data is continuously loaded and the software will require more memory.

  With the option ***Keep image in memory*** deselected, the image data is only loaded by the software when specific operations (Detection, Adjust Contrast, 3D view, Profile) require the use of raw image data. This gives you the possibility to optimize the use of memory available to the software, but some functionality (Pixel Intensity in the Cursor Information Window and Status Bar) will not be available unless you activate Adjust Contrast or Profile at the same time.

3 Image Pool

3.1 Introduction

This chapter is about opening and processing images in the Image Pool. You must be familiar with the concepts described in the last chapter (the Workspace, sheets, panes and images) in order to get the most out of it.

3.2 Start with good images

Your gels must first be converted into an image file by an appropriate imaging device. During digitization, a gel is resolved into a two-dimensional matrix of squares, or pixels. Each pixel in the generated image file is characterized by its X and Y coordinates, and its signal intensity, or gray value.

To make any analysis meaningful, it is important to start with good quality image files. The following paragraphs give some helpful tips on what resolution, depth and image formats should be used to obtain the best possible results.

3.2.1 Resolution

The scanning resolution of a gel is critical as it influences the amount of visible detail in the image. A low resolution corresponds to a large pixel size or a small number of pixels (or dpi, dots per inch). When the image resolution is too low, individual spots cannot be distinguished. On the other hand, when the scan resolution is too high, the image file becomes very large. This slows down the gel analysis significantly. A resolution between 150 and 300 dpi is generally sufficient for gel analysis.

3.2.2 Image depth

The range of potential gray levels in an image varies according to the image depth. In the case of an 8-bit image, one pixel has 256 (2^8) possible gray values (0 to 255). Images scanned with a higher image depth contain more information. A 16-bit image (2^{16} = 65536 gray levels) will reveal more subtleties. We strongly recommend an image depth of at least 12 bits for gel analysis. 16 bits is preferred.

Please make sure to use gray scale images for your analysis, and not color images. The extra color information (one intensity value for each color channel) is of no value.

3.2.3 Calibration

Some imaging devices can measure more gray levels (e.g., 100000 or more) than can be stored in the availabe image formats. These instruments encode the gray values using a nonlinear calibration curve (generally encoding low intensity values with higher accuracy than the high intensity values), to conserve as much information as possible in the saved files. Melanie uses the calibration curve contained in these image files to recalculate the measured gray values and use them for display and quantification.

Other devices convert raw pixel values into real-world units (generally optical density or OD). This means that the range of gray values is the same no matter what the original image depth. There are image capture devices, such as the GE Healthcare ImageScanner™ in conjunction with the LabScan software, that even allow you to perform this type of calibration. When this is the case, Melanie takes into account the conversion tables or calibration formulas stored in the files. You can also calibrate such an image capture device within Melanie using calibration step wedges or calibration strips.

3.2.4 Image editing

General purpose graphics software such as Adobe Photoshop™ ignore or even remove calibration information. Therefore, you should not use them to flip, rotate, crop or invert your images. Instead, use the software that came with your scanner or the dedicated tools in Melanie.

3.2.5 File format

The supported input formats are GEL (Molecular Dynamics), MEL (Melanie/ImageMaster™), TIFF (Tag Image File Format), IMG (Fuji), GSC and 1SC (Bio-Rad). Please note that the default TIFF format does not include calibration information, although some imaging tools do export .tif files that contain calibration tags.

3.2.6 DIGE file naming convention

To facilitate the import of DIGE images, it is recommended that the file names for the group of two or three images contain a common string and their respective dye names (Cy2, Cy3, Cy5).

3.3 Preview images

3.3.1 Open images

You can open gel images that are in any of the above-mentioned input formats (.mel, .gel, .tif, .img, .gsc, .1sc). DIGE images can also be imported and automatically grouped by opening .ds files.

To open gel images:

1 Do one of the following:

- Choose ***File > Open***.
- In the Workspace window, click the ***Project*** icon. In the Add Files window, search for ***Files of Type "All Image Files (*.mel; *.tif; *.gel; *.img; *.ds)"***.
- In the Navigator of the Workspace window, right-click on the ***Image Pool*** folder and select ***Add***.

2 In the Open Files or Add Files window, browse the directory where the image file is located, select its name and click ***Open***. Use the Shift or Ctrl keys to make multiple selections. Note that you can select files from multiple DIGE gels.

3 You are prompted to:

- **For non-DIGE images**: Specify the staining. If the staining cannot be found in the list, you can type a new one. The staining entered is only informative and does not influence the analysis, unless you assign the Cy2, Cy3 or Cy5 stains, in which case the images are treated as DIGE images. Click ***OK***.
- **For DIGE images**: Select the images that are part of the same DIGE gel (Figure 3-1). By default, the software proposes image combinations based on the file names. Click ***Add*** to confirm one DIGE gel at a time, or ***Add All*** if the proposed combinations are all correct. The suggested names for the created DIGE gels must be edited and confirmed individually.

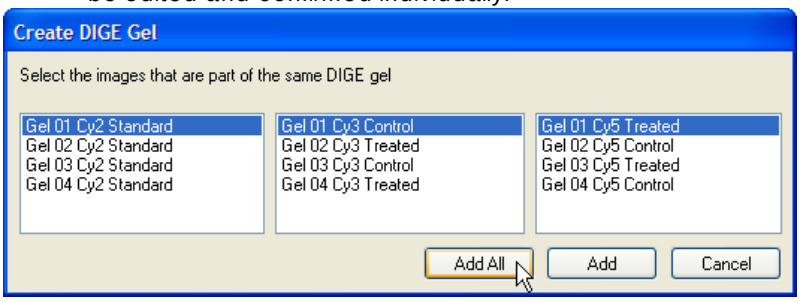


Figure 3-1. Create DIGE Gel window. The first list contains the Cy2 images to be opened, the second the Cy3 images and the third the Cy5 images. The software proposes the combination of images based on the file names.

Please note that the software checks the image resolution before opening a file. If the resolution is too low, you get a warning message. If it is higher than 225 dpi, you are able to scale the image in order to reduce its size.

3.3.2 Image pool sheet

The gel images appear in the Image Pool folder in the Workspace and are automatically opened in the Image Pool sheet (Figure 3-2). Non-DIGE images all appear in a single pane with the name Files. A separate

pane is displayed for each DIGE gel, containing the images belonging to that gel.

Remove

Gels remain in the Image Pool until they are added to a Project. When you are finished working with an image, right-click on it and select ***Remove***. To remove all images in the Image Pool, right-click on the Image Pool folder and select ***Remove All***. Please note that this action never affects the original image files (which always remain unchanged in their original location).

Hide / display

If you do not want to display all gels in the Image Pool sheet, you can right-click on certain items in the Image Pool folder and select ***Hide***. To show a hidden image, right-click on it and select ***Display***.

To open the Image Pool sheet at any time, right-click on the Image Pool folder in the Workspace and select ***Display***.

Properties

To rename a gel or image, or to see the file path, right-click on a gel and select ***Properties***.

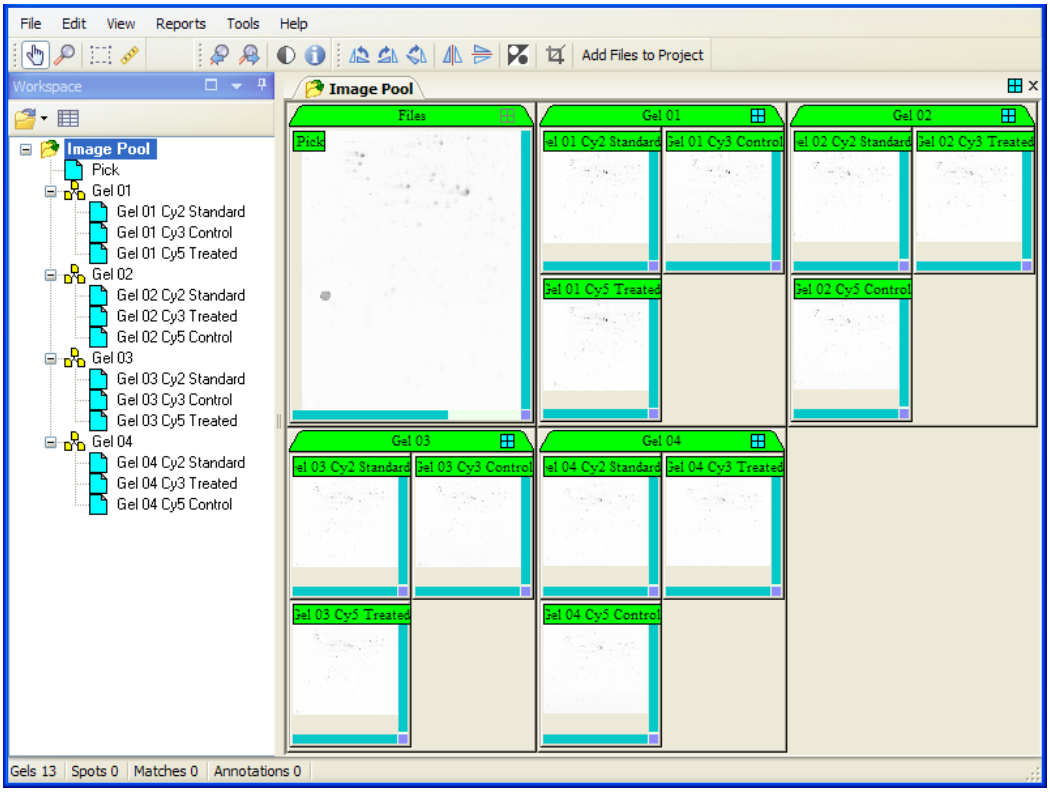


Figure 3-2. Image Pool folder in the Workspace and Image Pool sheet.

3.4 Process images

The Image toolbar provides the basic tools to process images: Rotate, Flip, Crop and Invert Gray Levels, which can also be found in the ***Edit > Gels*** menu. These tools are only available when the Image Pool is the current sheet. They are also optional and should only be applied when needed.

Processing your images in Melanie does not affect your original image files. Copies are saved in a temporary folder until the images are added to a project and saved in the corresponding project folder.

Please note that these operations are simultaneously carried out on the two or three images in a DIGE gel.

3.4.1 Rotate

Selected images can be rotated by 90° CCW (counterclockwise) or 90° CW (clockwise). Free rotation (the third icon) can also be applied, although it should be avoided since it modifies the original data.

To freely rotate an image:

1 Select the gel image to be rotated in the Image Pool sheet.

2 Click the ***Free Rotate*** icon.

3 A red grid appears on the image. The bold horizontal grid line plays the role of landmark to help you visualize the rotation. It becomes the new horizontal in your rotated image.

4 Click anywhere in the image and rotate the grid while holding the left mouse button. Release the button when the bold line is parallel with what should be the new horizontal in your image (Figure 3-3). You can also manually enter a rotation angle in the Rotation Tool window.

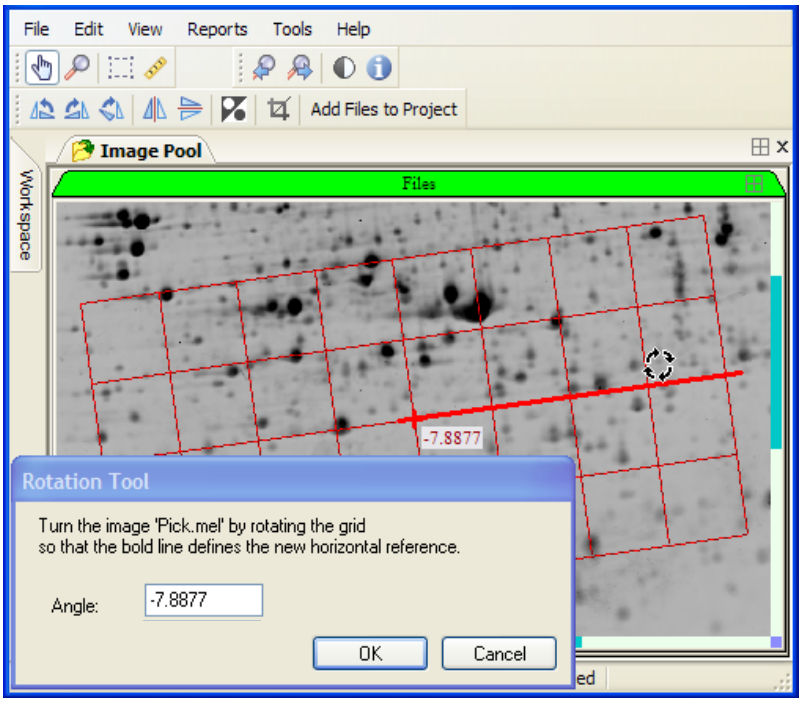


Figure 3-3. Rotation Tool window. The grid is rotated until its bold line is parallel with what should be the new horizontal reference. When the mouse button is released, the gel image is rotated.

3.4.2 Flip

When gel images are scanned in the wrong direction, you can Flip Horizontally or Flip Vertically to produce their correct mirror image.

3.4.3 Crop

You can crop your gel images with the ***Crop*** tool. This creates new gels that only contain the selected area and removes the outer area.

When you crop a gel, you get the choice to create a new image (a number is appended to the existing name) or to overwrite the image in the Image Pool (remember that gels in the Image Pool are copies of the original image file).

Crop area

The crop area is a region than can have an anchor attached to it. You can position the anchor on an easily recognizable protein spot. As the region moves with the anchor, and vice versa, you can easily crop a similar part of each gel by correctly positioning the crop areas (of the same size) in the gels (Figure 3-4).

To define a crop area:

1 Click the ***Region*** tool and place the cursor at the top left position of the area you want to crop. Hold down the left mouse button and move the cursor to the bottom right position (a dashed box is displayed). Release the mouse button at the end point.

2 Move a crop area by clicking inside the box and dragging it. Change the size of the area by dragging a corner or edge. To remove an area, double-click on the image.

3 If you want to attach an anchor to the crop area, hold the Alt key while clicking on an easily recognizable protein spot. A dark blue circle will be centered on the spot. Note that this anchor may be located inside or outside the crop area.

4 To change the position of the anchor, hold the Alt key and click another spot. To remove the anchor, hold the Alt key and click on the anchor.

5 Propagate the crop area to the other images in your sheet by holding the Shift key while clicking in the crop area.

6 Adjust the position of the crop area in each gel by moving it so that the anchor is centered on the appropriate spot.

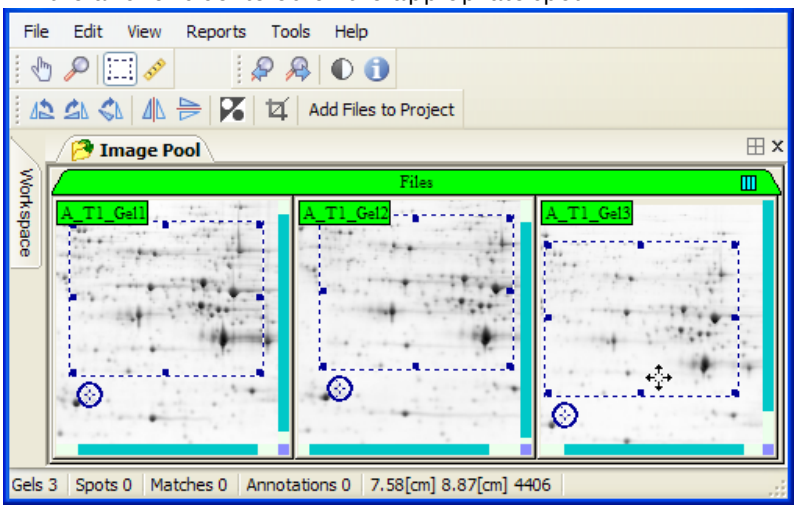


Figure 3-4. Identical crop areas in three gel images.

Crop area export / import

Crop areas can be exported and imported to ensure that the final size of all your cropped gels is identical, even between work sessions.

To export a crop area:

1 With the ***Region*** tool, define a crop area.

2 Select the gel in which you defined the crop area.

3 Choose ***Edit > Gels > Crop Area > Export*** to save the crop area to a file with the extension .cpt (Crop Tool).

4 Browse to locate a folder and enter a file name. Click ***Save***.

To import a crop area:

1 Select the gels to be cropped to a previously defined crop area.

2 Choose ***Edit > Gels > Crop Area > Import*** and select the previously saved file. Click ***Open***.

3 The crop area appears on the selected images.

4 By clicking inside the area with the ***Region*** tool and dragging it, you can move the crop area to superimpose the anchor on a characteristic spot.

3.4.4 Invert gray levels

You can invert the gray levels of selected gels. This means that if your image shows white spots on a black background, the inversion displays black spots on a white background (the required mode for analysis in Melanie).

CAUTION! When your images open in the software with white spots on a black background this may indicate that you used incorrect scanner settings or that your files are not properly imported. Please verify your image acquisition parameters. If you think the software does not correctly support your files (i.e., saved in one of the recommended input formats), please contact our technical support service.

3.5 Calibrate images

3.5.1 Display calibration information

It is recommended to use calibrated images for your gel analysis. To verify if your images were calibrated, and possibly view the calibration information:

- Choose ***Reports > Gel Table***, click the ***Settings*** icon and select the Calibration template from the ***Load*** drop-down list. The Calibration Unit, Formula, Name, Creator and Date are displayed in the report. If nothing appears in these columns, the gels were not calibrated.
- If your image was calibrated with LabScan 5.0 or 6.0, you can select the image and choose ***Edit > Gels > Show Calibration Plot*** to view the calibration curve. See below for details about the calibration curve.

If you are digitizing your images using a flatbed document scanner and do not have calibrated images yet, you can carry out and apply a

calibration at this point, before adding your images to a project. Use a scanned calibration step tablet to assign optical density (OD) values to the measured pixel intensities, and ensure that darker material is measured in the correct proportions to the lighter material.

It is generally recommended to perform this type of intensity calibration on the image capture device once every month.

3.5.2 Create a calibration

Calibration step tablet

To calibrate the image capture device, you need to scan a calibration step tablet or calibration strip along with your gels. These step tablets have known intensity values (expressed in optical density, OD, or diffuse density, DD) published by the manufacturer of the step tablet.

Please note that for the purpose of 2D gel analysis, it is only useful to calibrate the image capture device when working in transparent mode. No calibration needs to be done when you do reflective scanning. When both a transparent and a reflective calibration strip are provided, be sure to use the appropriate calibration step tablet.

Calibration tablet file

The OD values for the step tablet have to be specified in a Calibration Tablet File, together with other information such as the height and width of the tablet, and the number of steps.

An example of such a Calibration Tablet File (Kodak2.tab) can be found in the Template\Tablet folder of the Melanie installation directory. This Calibration Tablet File is made for use with the Kodak Step Tablet no. 2. If you do not use this specific step tablet, you can copy the file and edit the data to make your own Calibration Tablet File. You can edit the file with tools such as Windows Notepad.

As the intensity values supplied with your step tablet are generally expressed in diffuse density (DD), you have to convert them to OD values. For this purpose, the manufacturer of the step tablet should provide the appropriate relationship. For the Kodak Step Tablets no. 2 and 3, for example, this is OD = 1.4 DD.

Create calibration

You can create a calibration once you have scanned the step tablet and have a correct Calibration Tablet File.

To create a calibration:

1 Open the step tablet image file. If necessary, rotate the image so that the light steps are displayed at the top.

2 Choose ***Tools > Calibration Tablet > Create***.

3 In the Load Step Tablet Definition window, browse to the folder where you saved the Calibration Tablet File (.tab) specifically tailored to your step tablet (see above), select the file and click ***Open***.

4 A red calibration step overlay appears on the image and the Create Calibration window is displayed (Figure 3-5).

5 Adjust the position of the steps by dragging the overlay while holding the left mouse button. The size of a step on the red overlay (the distance between two short horizontal lines) should correspond exactly to the size of a step on the image. If this is not the case, you must adjust the height of the tablet in the Calibration Tablet File.

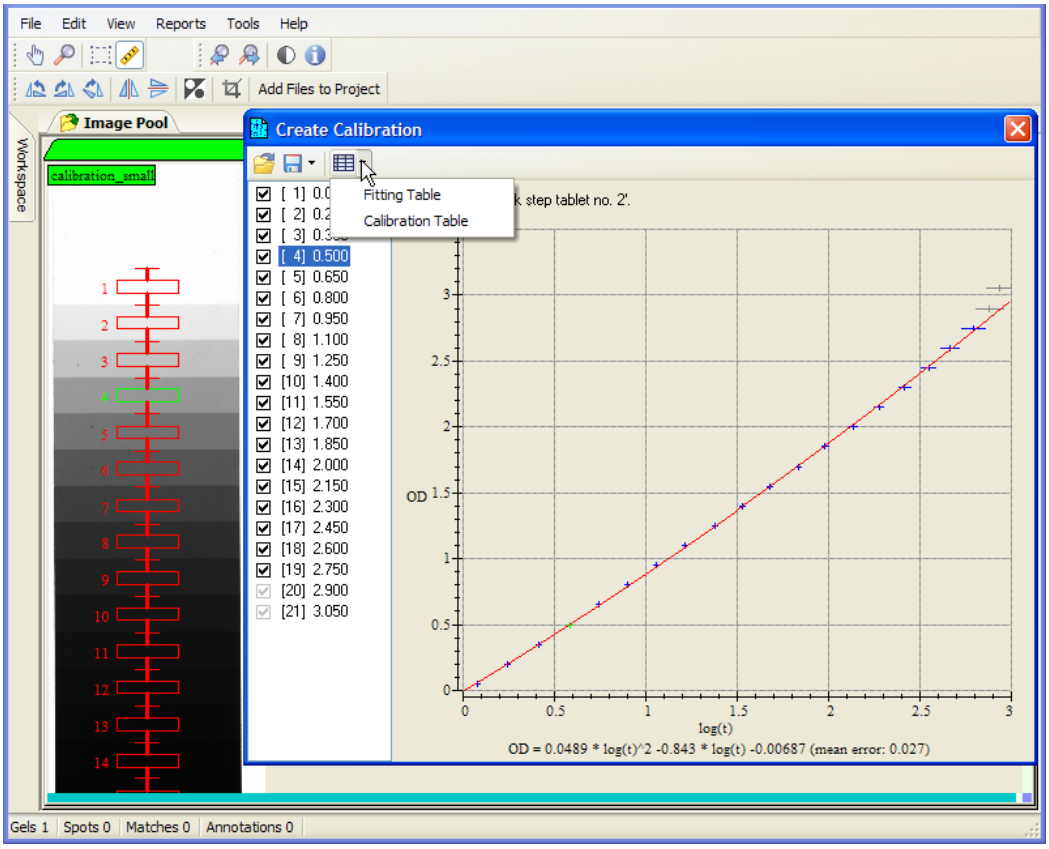


Figure 3-5. Image of the step tablet with the red calibration step overlay. The Create Calibration window shows the calibration curve and the OD values for the different steps (on the left). When selecting a step in the list, the corresponding step becomes automatically highlighted in green on the step tablet overlay and in the calibration graph.

6 At the left of the Create Calibration window, you see the theoretical optical density (OD) values of the different steps in the tablet (the values you entered in the Calibration Tablet File). Some steps are automatically deselected (grayed out) because of their unreliable values. You can deselect additional ones if you estimate that they should be excluded from the calibration process. At the

right of the Create Calibration window you see the calibration curve between the logarithmic transmittance values on the X-axis, and the OD intensities on the Y-axis. Note that the measured intensity values for each step are calculated as median intensities over all the pixels in the small rectangle area for each step (on the step overlay). The horizontal dispersion intervals in blue (or gray for deselected spots) represent the intensity ranges when 10% of the less intense and 10% of the most intense pixel values are removed. The calibration formula and error are given below the graph.

7 You can display some reports to judge the quality of your scanner calibration (see below for more details).

8 You can also choose whether you want to force the calibration curve to go through the origin (see Reports icon below).

9 Once you are satisfied with the calibration, close the Create Calibration window. The software asks whether you want to apply this new calibration. If you answer Yes, Melanie applies the calibration to the image.

The icons in the toolbar of the Create Calibration window and their use are:

Open another step tablet definition.
Save the calibration (with the extension .cal).
Print the calibration graph.
Copy the calibration graph to the clipboard.
Display related **reports**: • The ***Fitting Table*** displays the calibration formula. • The ***Calibration Table*** displays for each step: the step number, the measured average gray level, the theoretical intensity value, and the fitting error (difference between the curve and the point). • The ***Force Curve Through Origin*** option allows to force the calibration curve to go through zero.

3.5.3 Apply a calibration

Once you have created a calibration using a step tablet, and saved this calibration in the step tablet image file or a calibration file (.cal), you can apply the calibration to newly-scanned image files.

To apply a calibration to a gel:

1 Display and select the gels the calibration should be applied to.

2 Choose ***Edit > Gels > Apply Calibration***.

3 Select the source of the calibration information. This can be an open gel (or step tablet image) that was already calibrated or you can select a file (.cal or .mel) from the hard disk.

All pixel and spot values subsequently calculated and displayed in the software correspond to the calibrated values.

3.5.4 Remove a calibration

You can remove a calibration from a gel. Note that this can also be done for gels that were already calibrated when you imported them into the software.

To remove a calibration from a gel:

1 Display and select the gels from which you want to remove the calibration.

2 Choose ***Edit > Gels > Reset Calibration***.

3 Confirm your choice.

3.5.5 Control a calibration

The Control calibration mode allows you to verify whether you are using the correct calibration. It requires a different, specially calibrated step tablet (e.g., Kodak Step Tablet no. 3), which you compare to your previous calibration results. So you have a calibration step tablet for everyday use and a specially calibrated control step tablet to verify your calibration periodically.

To control a calibration:

1 Scan your control step tablet (e.g., Kodak Step Tablet no. 3) and open the image in Melanie. If necessary, rotate the image such that the light steps are displayed at the top.

2 Choose ***Tools > Calibration Tablet > Control***.

3 You are asked to load the calibration to be controlled. This calibration could have previously been saved using the ***Save*** icon in the Create Calibration window (.cal) or can simply come from a calibrated image file (.mel) such as the calibrated step tablet image obtained in the section above.

4 Next load the definition of the control step tablet. This Control Tablet File must be specifically adapted to this new step tablet. That is, it should have been edited with a tool such as Windows Notepad so that it contains the appropriate OD values, height, width, and number of steps corresponding to the control step tablet.

5 The red calibration step overlay appears on the image of the control step tablet and the Control Calibration window is displayed.

6 Select the ***Spot*** or ***Annotation*** tool in the Melanie toolbar and adjust the position of the steps.

7 You should now verify that the calibration curve is passing through the data points correctly and with minimum dispersion intervals. If this is not the case, try to find out why your current calibration does not seem to work properly.

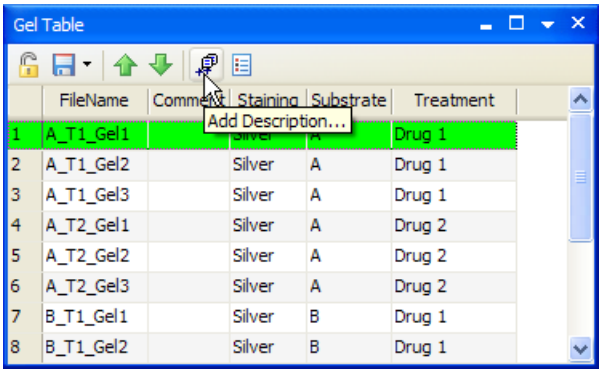
3.6 Describe images

3.6.1 Gel descriptions

A gel description is a kind of label for the gel image. You can enter information about your gel images to be used for later reference by yourself or any colleagues. This information can include sample type, gel running protocol, date of the experiment, operator name, pH range, SDS gel percentage, or anything else you can think of that would be useful to know about the images. All of this data is entered as Gel Descriptions.

When opening images in the software, you are asked to specify the staining. Staining is a standard Gel Description. This information is simply informative, i.e., it is not used by the software except when you specify the Cy™ dye for DIGE images that do not have this information in their file name.

Each image can only have one description of a given category. For example, a gel description category Treatment could contain the description “Drug 1” for certain images and “Drug 2” for other images (Figure 3-6). You can define any number of categories for an image or a given set of images.



	FileName	Comment	Staining	Substrate	Treatment
1	A_T1_Gel1		Silver	A	Drug 1
2	A_T1_Gel2		Silver	A	Drug 1
3	A_T1_Gel3		Silver	A	Drug 1
4	A_T2_Gel1		Silver	A	Drug 2
5	A_T2_Gel2		Silver	A	Drug 2
6	A_T2_Gel3		Silver	A	Drug 2
7	B_T1_Gel1		Silver	B	Drug 1
8	B_T1_Gel2		Silver	B	Drug 1

Figure 3-6. Gel descriptions in a Gel Table (using the Descriptions template).

3.6.2 Display gel descriptions

Gel descriptions can be displayed in a Gel Table. Select the predefined Descriptions template in this table to display only the columns for the gel description categories.

To display gel descriptions:

1 Choose ***Reports > Gel Table***.

2 Click the ***Settings*** icon.

3 In the Gel Table window, select the predefined Descriptions template from the ***Load*** icon drop-down list. The boxes for the FileName and all gel description categories (by default, only Staining and Comment) are checked.

4 Click ***OK***.

3.6.3 Edit gel descriptions

You can edit a gel description for one or more selected gels.

To edit gel descriptions:

1 Select the images for which you want to add or edit descriptions.

2 Choose ***Edit > Gels > Edit Description***.

3 In the Add Gel Description window, select an existing gel description category or create one. Click ***OK***.

4 Enter the gel description that applies to the selected images. Click ***OK***.

5 The new gel descriptions are displayed in the Gel Table.

Alternatively, you can define gel descriptions by clicking on the ***Add Description*** icon in the Gel Table toolbar. It's behaviour is identical to that of the ***Edit > Gels > Edit Description*** menu.

You can delete all gel descriptions of a certain category for the selected gels by choosing ***Edit > Gels > Delete Description***.

3.6.4 Permanent gel description categories

When gel description categories are created as described above, they are only used for the gels that were selected during the creation of the categories. To make gel description categories permanent in the software (always availabe from the category list), you must define them in the Options.

To create permanent gel description categories:.

1 Choose ***Tools > Options***.

2 Click the ***Add in the Gel Descriptions*** tab.

3 Enter a category name in the ***Add Category*** box and click ***OK***.

4 The category is displayed in the permanent category list.

To remove a permanent category, select it from the list and click ***Delete***.

4 Projects

4.1 Introduction

A project includes all gels, spots, matches, annotations, spot sets, and other information produced and analyzed during the course of a specific gel study. You can create or add many projects in the Workspace.

A project in the Workspace can include one or more match hierarchies (e.g., AB in Figure 4-1), each of which contains a Match folder and a Classes folder. The Match folder describes how gels or populations of gels, called match sets, should be matched together. The Classes folder is where the biological question is stated, through the definintion of classes of gels to be compared.

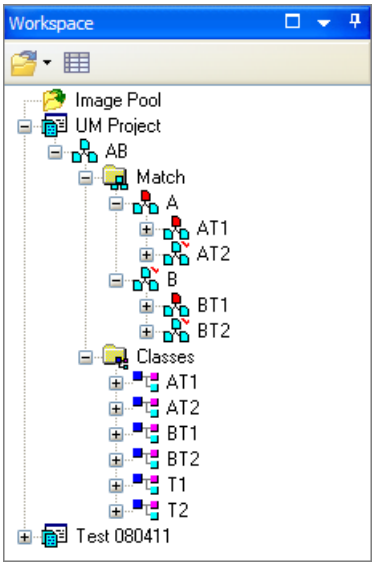


Figure 4-1. Project structure.

The next sections further explain the principles of match hierarchies and classes, and how they can be used.

4.1.1 Match hierarchies

All images in an experiment are not equally easy to compare, even when the gels are run in a highly controlled way. Typically, gels belonging to the same biological group are easier to match (i.e., corresponding spots are easier to find) than images from different biological populations.

It is therefore recommended to use hierarchical match structures to create more efficient match designs. Figure 4-2 and Figure 4-3 show an example of a match hierarchy, or root match set, AB and submatch sets A, B, AT1, AT2, BT1 and BT2. Gels or match sets with a red marker are used as the reference in the matching and always appear first in the list.

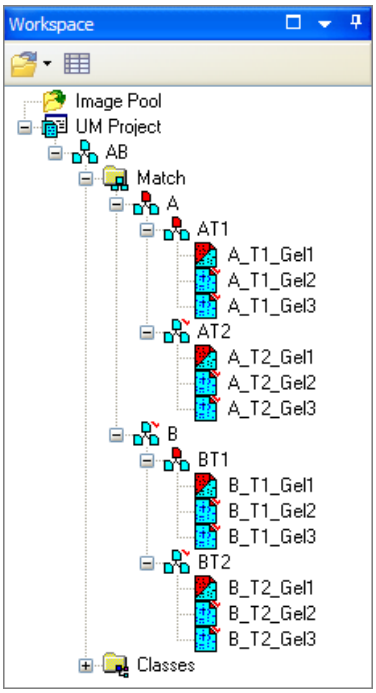


Figure 4-2. The Match folder. In this example experiment, a set of samples from bacteria were cultivated with either substrate A or with substrate B. Under both growing conditions, two treatments were tested. Therefore, 4 different populations exist. A gel was run for each of the 3 samples in a population, giving a total of 12 gel images.

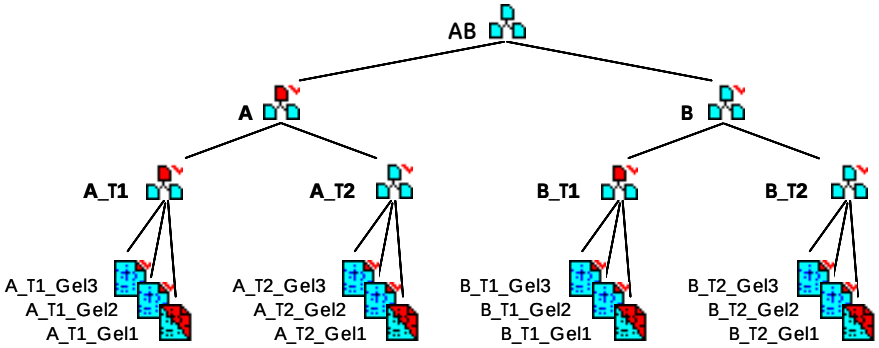


Figure 4-3. A tree view of the example match hierarchy described above.

An important advantage of this type of match hierarchy is that it minimizes the number of difficult match combinations. Instead of having to match 9 images (those belonging to AT2, BT1 and BT2) to a global reference (e.g., A_T1_Gel1) that is not belonging to the same population, only 3 slightly more complex match combinations must be performed (AT1 versus AT2, BT1 versus BT2, and A versus B). This significantly reduces time spent on match editing.

There is another important reason why it is useful to adopt hierarchical population matching instead of matching all images against a unique arbitrary reference image. Only spots matched with a spot in another gel are included in Gel and Class Analysis Tables (all spots are of course presented in the Spot Table). The likelihood of a spot being matched is much higher when matching with a gel from the same biological population. Spots that are represented in a single population (submatch set) are therefore included in the analysis, even if they are not in the global match reference. This considerably reduces the number of spots missed in the analysis.

CAUTION! DIGE gels are inherent match sets. A DIGE gel (the entity with its composing images) is treated as any other non-DIGE gel when setting up a match hierarchy.

Once created in the Workspace, you can display a complete match hierarchy (e.g., AB in Figure 4-2) in a sheet and carry out spot detection on all the included gels. After defining one or two landmarks, the entire experiment is matched in a matter of seconds, and matches are automatically propagated at each level of the match hierarchy.

4.1.2 Classes

In the Classes folder, you state your biological questions. This means that you define a class for each set of gels that you want to compare with other such entities. Your goal, by comparing classes and therefore the

gels within those classes, is to find the protein expression variations between different biological states.

The classes created in the Classes folder of a given match hierarchy can contain any of the images in that hierarchy. One image can be in different classes. In Figure 4-4, for example, the same 12 images can be compared as part of the 4 classes AT1, AT2, BT1 and BT2, or as part of the two classes T1 and T2.

You can define classes at any time, even in the very beginning of your gel analysis study when no spots are detected. To carry out statistical analysis, however, your gels must be detected and matched.

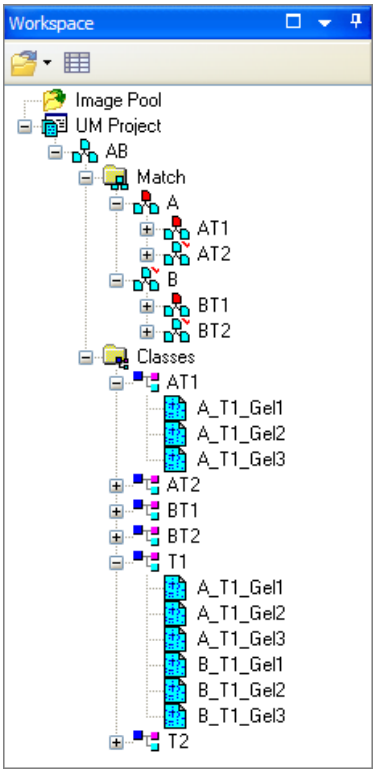


Figure 4-4. The Classes folder.

4.2 Create a project

4.2.1 The first time the software is launched

As long as no projects have been created or added to the Workspace (e.g., the first time you open the software), you are prompted to create

a project (see Figure 4-5). Enter a project name, browse to where the project folder should be saved, and possibly add a comment.

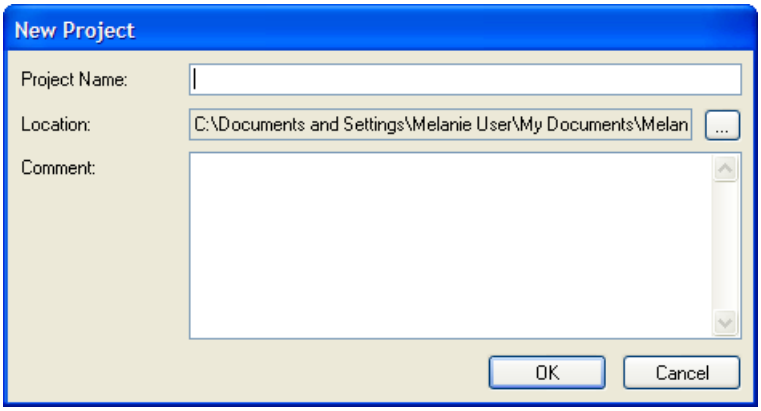


Figure 4-5. New Project window.

4.2.2 At any time

You can create a new project at any time by selecting ***New*** from the ***Project*** icon drop-down list in the Workspace toolbar (Figure 4-6). Again, enter the ***Project Name***, ***Location*** and ***Comment*** (Figure 4-5).

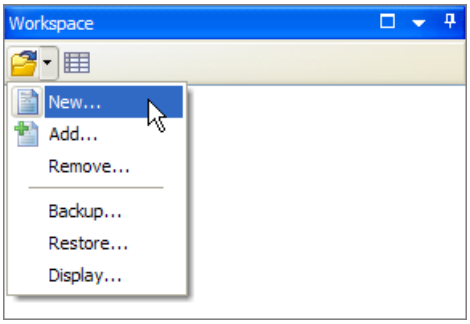


Figure 4-6. Project icon drop down menu in the workspace.

4.2.3 Add files to project

Use the ***Add Files to Project*** icon in the Image toolbar to add gels from the Image Pool to a project (Figure 4-7). There is the possibility to create

a project in the Add Files to Project window. An existing or new match set name must be entered as well.

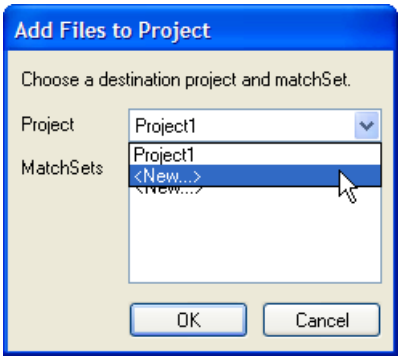


Figure 4-7. Add Files to Project window. A project can be created by choosing <New...> from the Project list.

4.3 Create match hierarchy

4.3.1 Create a match set

The easiest way to create a match hierarchy is by adding gels from the Image Pool to a project. The idea is to select only the gels that should be added to a particular match set (e.g., gels from the same population or same experimental batch) and then to create this match set. Different options exist:

- Select gels in the Image Pool folder and drag them onto a project name. Enter a name for the new match set.
- Select gels in the Image Pool sheet and drag them onto a project name in the Workspace. Enter a name for the new match set.
- Select gels in the Image Pool sheet and click ***Add Files to Project*** in the Image toolbar. Set or create the destination project, and then click ***<New...>*** in the MatchSets field (Figure 4-8). Enter a name for the new match set.

Alternatively, create an empty match set by right-clicking on the project name and selecting ***Create MatchSet*** in the contextual menu. Drag images from the Image Pool (folder or sheet) into the new match set. Gels selected in the Image Pool sheet can also be added to an existing match set by clicking the ***Add Files to Project*** icon.

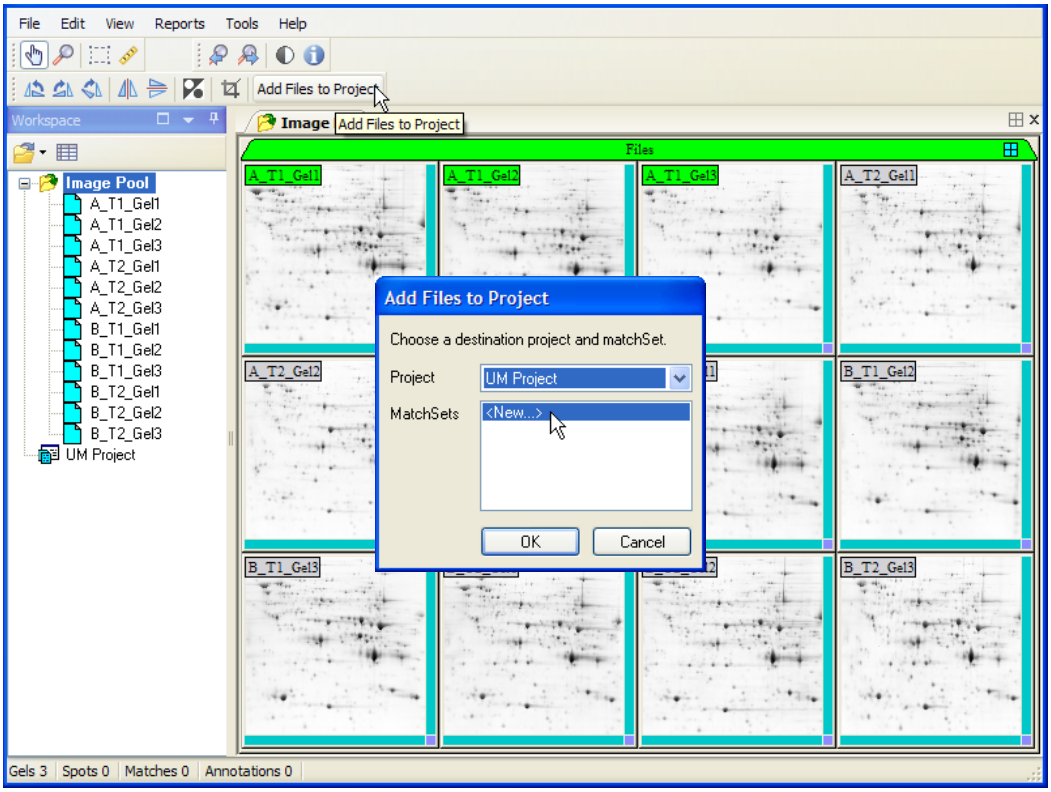


Figure 4-8. Add Files to Project. The three images of population A_T1 are selected in the Image Pool sheet. After having clicked the Add FIles to Project icon, the destination project and new name for the MatchSet can be set.

4.3.2 Merge a match set

Once match sets have been created, they can be merged into higher level match structures for further matching. Select the match sets, right-click on one of them, choose ***Merge MatchSet*** from the contextual

window, and give a name for the new match set (Figure 4-9). A hierarchy (Figure 4-2) can be created.

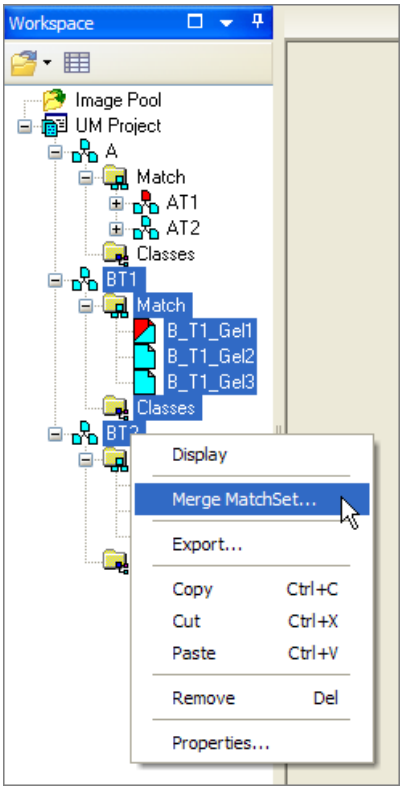


Figure 4-9. Match sets BT1 and BT2 are selected for merging. Match sets AT1 and AT2 were already merged this way into a match set A.

4.3.3 Set reference

Within each match set, the gel or match set that has a red marker and appears first in the list is used as the reference in the matching process. To change the match reference, drag the desired gel or match set onto the name of its parent match set so that it moves into the first position.

> **CAUTION!** You can change the match reference as long as the images in your match set have not been matched. Once they are, the reference image can no longer be changed.

The reference for each match set must be carefully chosen. This is because automatic matching compares the spots in the reference to those in the other images. If a spot is absent from the reference, it cannot be matched automatically (although it can be matched

manually with spots in other gels). The rule of thumb is to choose the gel or match set with the most and the best quality spots as the reference.

4.3.4 Use existing match sets

You may wish to carry out several analyses, using different matching schemes. You can copy match sets to use them in another configuration. In Figure 4-10, for example, the match sets A_T1, A_T2, B_T1 and B_T2, were copied to be used in populations per treatment (T1, T2) rather than growing substrate (A, B). Because existing matches are conserved when copying match sets, this can save a lot of work.

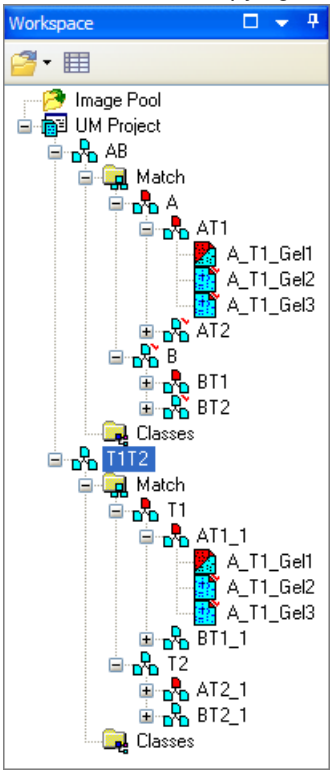


Figure 4-10. Match sets AT1, AT2, BT1 and BT2 were copied to be used in the match hierarchy T1T2.

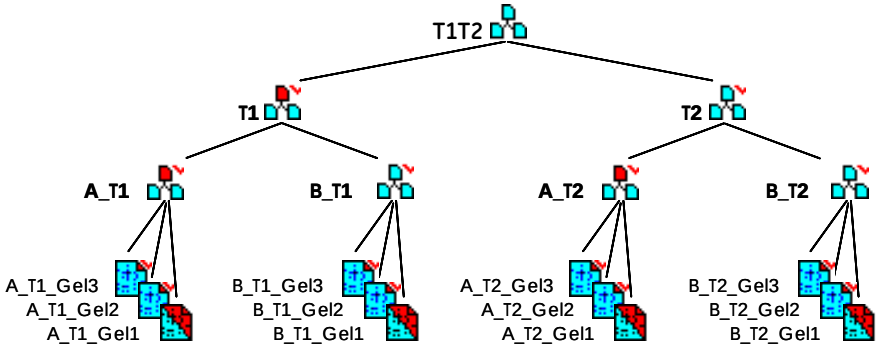


Figure 4-11. A tree view of the match hierarchy in Figure 4-10. The match sets A_T1, A_T2, B_T1, and B_T2 have been reused in a different configuration compared to the example in Figure 4-3.

You can simply drag one or more match sets to a new destination, or copy (right-click on the match set) and paste them in the new destination (right-click on the destination project or match set). The destination can be a match set or open project. Copies of the match sets, including the existing matches, are created. A number is appended to the orginal name. You can then rename the copied match sets (see below).

To copy a match set in the same project, hold down the Ctrl key while dragging the match set onto the project name.

4.3.5 Export / import a match set

The easiest way to provide others with access to the data is to export a match hierarchy (the match set that is the parent of the project, containing the folders Match and Classes). Simply right-click on the parent match set and choose ***Export MatchSet***. The entire match set (including images, matches, spots, annotations, spot sets) is compressed into a single .exp file.

A .exp file can be imported into a project by right-clicking on the project name and choosing ***Import MatchSet***.

Note that if the project data are saved in a folder on a shared network, colleagues having access to this folder can open and work with the project, and therefore the match sets.

4.4 Create classes

4.4.1 Create a class

In the Match folder of a hierarchy, select all images that should be added to a single class (group of biologically-related images). You can select match sets as well. However, only one type of item (images or match sets) should be selected at a time.

- **Option 1**: Drag the selection onto the Classes folder of the same hierarchy. Enter a name for the new class and click ***OK***. You can also use the Copy and Paste options in the contextual menus.
- **Option 2**: Right-click on one of the selected items and choose ***Add In Class***. Enter a name for the new class and click ***OK***.

Alternatively, create an empty class by right-clicking on the Classes folder and selecting ***Create Class*** in the contextual menu. Drag images or match sets into the new class.

4.5 Handle project items

Several operations are available for most items in a project, mostly by right-clicking in contextual menus.

4.5.1 Display

To display one or more images, match sets or classes in a new sheet, right-click on a selected item and choose Display. Please note that only complete match sets or classes are displayed. This means that if you select one image in a match set, all images in the match set are shown.

In the resulting sheet, only the highest level and lowest level items (match sets or classes) are specifically visualized, in the sheet and panes, respectively. To select intermediary levels, you can use the corresponding icon in the pane tab of the match reference for that level.

Once the images are displayed in a sheet, you can start working with them. You can change the layout settings to focus on certain images and hide others.

4.5.2 Remove

To permanently remove an item from the project, select ***Remove*** in its contextual menu. This will delete the item from your hard disk.

4.5.3 Properties

When choosing ***Properties*** for an item, certain of its attributes are shown such as its name, creator, file path, etc. Here is where you can change the name of an item or enter a comment to describe the item.

4.5.4 Move

You can rearrange your images, match sets and classes to change their position in the list or move them into another match set or class. Just drag your image, match set or class to the desired position. It is generally inserted after the item you drop it on. Whenever there is a possibility to insert it inside or after an entity, you are asked to specify.

4.6 Save projects

4.6.1 Project folder

When you create a project, you are asked to specify a name and a location on your hard disk. All the data related to the project (images, spots, matches, annotations, spot sets) are saved in this location, in a folder with the name of the project. All users that have access to this folder are able to open, view and work with the project.

The project folder contains the following files and folders:

- ***Projectname.prj***: This project file is the link between all the other data files in the project folder. If you want to add an existing project to your workspace, you must search for, and open, its .prj file.
- ***Raw Images***: This folder contains the raw image files in .mel format.
- ***MatchSet***: This folder contains the gel (.gda) and match (.mda) data in subfolders. The .gda files are used to store the spot information. The .mda files contain the match information.

4.6.2 Save

The software automatically saves your work when you close a sheet or exit the software. You can also save your work by choosing ***File > Save***. This saves all your data in the corresponding project folder on the hard disk.

4.6.3 Copy

You can make a copy of a project by selecting ***Copy*** in the ***Project*** icon drop-down list in the Workspace toolbar. This allows you to make modifications on the copy without altering the original project.

4.6.4 Share project

If the project data are saved in a folder on a shared network, colleagues having access to this folder can open and work with the project. The project must be added to the colleague's Workspace using the procedure described below.

4.7 Manage projects

4.7.1 Add / remove projects

Remove

You can remove a project from your workspace by selecting ***Remove*** in the ***Project*** icon drop-down list in the Workspace toolbar. Select the project(s) to be removed and confirm. This does not delete the project folder from your hard disk.

To permanently remove a project folder, you must delete it from your hard disk. Make sure that this folder is not accessed by colleagues in your network before proceeding.

Add

A previously removed project can be re-inserted at any time. Another user can also add it to his/her workspace.

To insert an existing project:

1 Select ***Add*** in the ***Project*** icon drop-down list in the Workspace toolbar.

2 In the ***Add Files*** window, browse the directory where the project file (.prj) is located, select its name and click ***Open***.

4.7.2 Add files to project

Please note that ***Add*** in the ***Project*** icon drop-down list in the Workspace toolbar also enables you to add project elements instead of entire projects. You can open the following file types:

- Project (*.prj)
- Project Backup (*.bkp)
- Export File (*.exp)
- All Image Files (*.mel; *.tif; *.gel; *.img)
- MatchSet Data (*.mda)
- Gel Data (*.gda)

4.7.3 Backup / restore project

It is good practice to do regular backups so that you can recover your work at any time. With the Backup function in the Workspace, one or more project(s) can be archived by writing all project-related data (images, spots, matches, annotations, spot sets, enabled spots) into a single compressed file with the extension .bkp. This file can then be restored when needed.

To backup a project:

1 In the Workspace, select ***Backup*** in the ***Project*** icon drop-down list.

2 Select one or more projects to backup, and confirm when prompted.

3 In the Backup Project window, browse to the directory you want, enter a file name and click ***Save***.

4 The backup file (with the extension .bkp) is archived.

To restore a project:

1 In the Workspace, select ***Restore*** in the ***Project*** icon drop-down list.

2 In the Restore Project window, browse the directory where the backup file is located, select its name and click ***Open***.

3 Select the project(s) to restore from the list. Use the Ctrl or Shift keys to make multiple selections. Click ***Restore***.

4 If the project already exists in the Workspace, there is the possibility to replace it with the archived project. If the project is not present in your workspace, you are given two options for restoring a project. By restoring to the original file path, the files are put back in the original project folder. By restoring to a new file path, the project is replicated in a new location, which must be specified. Click ***Restore***.

4.7.4 Project visibility

When there are several projects in the Workspace, it can be useful to temporarily hide some of them. Select ***Display*** in the ***Project*** icon drop-down list and check (or uncheck) the Visibility box in front of the projects that should be hidden (or shown).

4.7.5 Project properties

To quickly view details (creator, comments, etc.) about a project, right-click on the project and select ***Properties*** in the contextual menu.

You can also modify the Project Name and Comment.

5 Gels

5.1 Introduction

This chapter presents operations that specifically relate to gel images. You will learn how to manipulate images, use different tools to view signal intensities, and discover ways to visually compare images. The last section explains how to save, export and print gel images.

5.2 Manipulate images

The main toolbar provides the following tools to deal with images :

5.2.1 Move

Select this tool, click in the image and hold down the left mouse button while moving the cursor. The image changes position. Release the button at the position you want.

Apply to all gels

To move all gels in the current sheet by the same displacement, hold down the Shift key while changing the position in one of the gels.

Double-click

To move all gels in the current sheet to the same position with the same zoom factor, double-click on one of the gels. The corresponding position in the different gels is estimated by interpolating between the surrounding matches, or if no matches exist, between the two nearest landmarks. Finally, when no landmarks exist, the gels are aligned at the same location using the X and Y coordinates.

Alternatives

There are other ways to change positions in gels:

- Use the ***View > Gels > Navigation > Move*** menu.
- Use the shortcut keys for the above-mentioned menu commands.
- Use the scrollbars.

5.2.2 Zoom

Select this tool, click repeatedly in the area of the gel where you want to zoom in. Right-click repeatedly on the gel to zoom out.

You can also define a zoom area: place the cursor at the top left corner of the area, hold down the left mouse button, and move to the bottom right position (a red box is displayed). Release the mouse button at the end point.

Apply to all gels

To move all gels in the current sheet to the same position with the same zoom factor, hold down the Shift key while zooming in or out on one of the gels.

Alternatives

There are other ways to zoom gels:

- Use the mouse scroll wheel to zoom in or out.
- Use the ***View > Gels > Navigation > Zoom*** menu.
- Use the shortcut keys for the above-mentioned menu commands.
- Use the scrollbars.
- To temporarily enlarge an area in a gel image, hold down the Ctrl key while the ***Zoom*** tool is activated. The area under the cursor is enlarged as if you were looking through a magnifying glass.

Overview option

When you zoom in on a gel, it can be helpful to have an overview of where the region is localized on the full image (Figure 5-1). This overview enables you to easily locate and move to any region you want on your gel.

Choose ***View > Global > Show Overview*** to show or hide the overview of each image in its lower right corner. The green rectangle in the overview corresponds to the current view of the gel. You can drag the green rectangle to another position to display a new region.

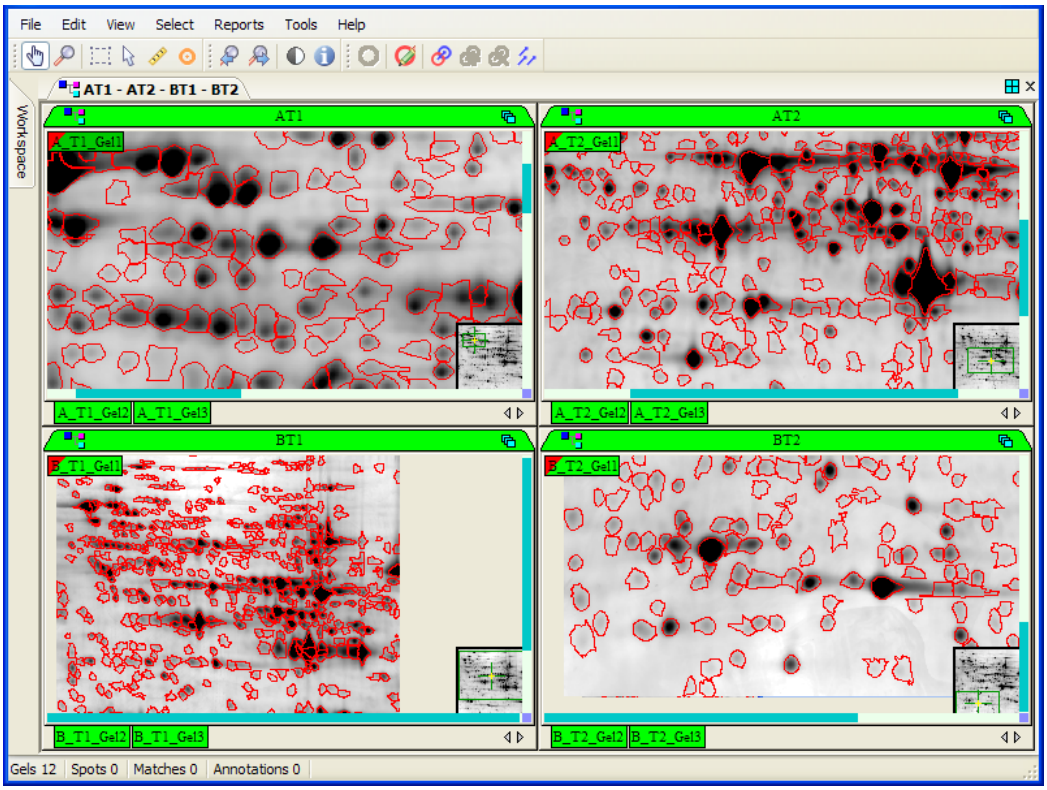


Figure 5-1. Overview option activated. In the lower right corner of all images in the Display Zone is a small overview of the entire gel with a green rectangle corresponding to the visible gel area.

5.2.3 Region

A region is a rectangular area in an image that is of interest for displaying a 3D View, for previewing spot detection parameters or adjust contrast settings, for cropping, etc.

Select this tool, place the cursor at the top left corner of the area you want to define, hold down the left mouse button, and move to the bottom right position (a dashed box is displayed). Release the mouse button at the end point (Figure 5-2).

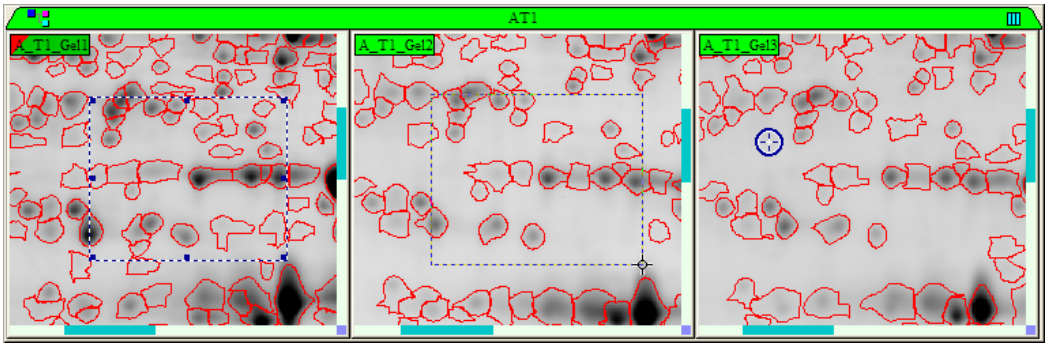


Figure 5-2. Region tool. The left gel shows a region after selection, the middle gel during selection, and the right gel shows when the region has been reduced to its minimum size.

Edit region

You can move a region by clicking inside the box and dragging it. You can also change the size of the box by dragging a corner or edge. To remove a region, double-click on the gel. Please note that if the box is reduced to its minimum size, then its appearance changes to a blue circle.

Apply to all gels

To define the same region on all gels in the current sheet, hold down the Shift key while drawing the box on one of the gels or while clicking in an existing region.

5.2.4 Measure

Select this tool to measure pixel, pI, MW, or real world (centimeter or inch) distances between two pixels in an image. Click on a pixel (e.g., center of first spot), hold down the left mouse button and move to the pixel (e.g., center of second spot) for which you want to measure the distance. The horizontal and vertical distances between the start and end points are displayed (Figure 5-3).

The units of the displayed coordinates can be changed by choosing ***Tools > Options*** and looking under the ***Display*** tab.

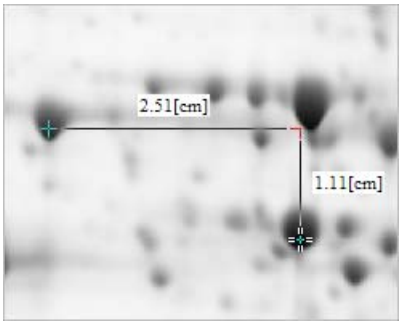


Figure 5-3. Measured distance between pixels on an image. In the example, the horizontal and vertical distances between the starting (left) and end (right) points are 2.51 cm and 1.11 cm, respectively.

5.2.5 Bookmarks

You can bookmark a view of your images in a sheet. This saves the currently visible areas. Bookmarks allow you to return to the same area on your gels at a later time or to show a similar area on the same images opened in a different sheet.

Create
Choose ***View > Sheet > Bookmarks > Create*** and enter a name.

Load
To go back to previously visible areas, choose ***View > Sheet > Bookmarks > Load*** and select the name of the corresponding bookmark. Select the name of another sheet in the list to reproduce the visible areas of that sheet in the current sheet.

Delete
To remove a bookmark from the list, choose ***View > Sheet > Bookmarks > Delete***.

5.3 View signal intensity

The digitized image is composed of individual pixels, each of which is characterized by its X and Y coordinates, and its signal intensity (raw pixel value). This section describes different approaches to explore the signal intensity and adapt the way it is visualized.

5.3.1 Adjust contrast

Sometimes the gray levels displayed by default are so low that small spots are hardly visible. To emphasize these very faint spots, you can adjust the contrast of the image and/or display images using pseudo colors.

Choose ***View > Gels > Adjust Contrast*** and select a region of interest on one or more images using the ***Region*** tool. These regions will allow you to preview the contrast and pseudo color settings before applying them to the selected images. The size and position of the preview regions can be adjusted at any time. After having adjusted the settings as described below, click ***Apply***. The new contrast settings are saved with the image file.

CAUTION! Any Adjust Contrast changes only influence how the image is displayed on your screen and do not affect the underlying data, spot detection, and quantitation.

Gray level mapping

Some scanners are able to scan 2-DE images with 100000 gray levels or more. Because common computer screens are only able to display 256 gray levels, mapping must be undertaken between the image gray levels and the 256 screen gray levels. By default, the software uses a linear mapping function where the lightest point in the image is mapped to 0 (white) and the darkest point is mapped to 255 (black).

This is illustrated in Figure 5-4. The histogram displays the frequency with which each gray level (from 3524 to 28530) occurs in the first image of the sheet. The low gray levels (on the left of the histogram) corresponding to the background occur very often, whereas the high gray levels (on the right of the histogram) corresponding to the darkest spots are much less frequent. The red line indicates that the minimum and maximum gray levels (3524 and 28530, respectively) are remapped by the default linear mapping. The vertical axis for the red remapping graph corresponds to the 256 screen gray levels.

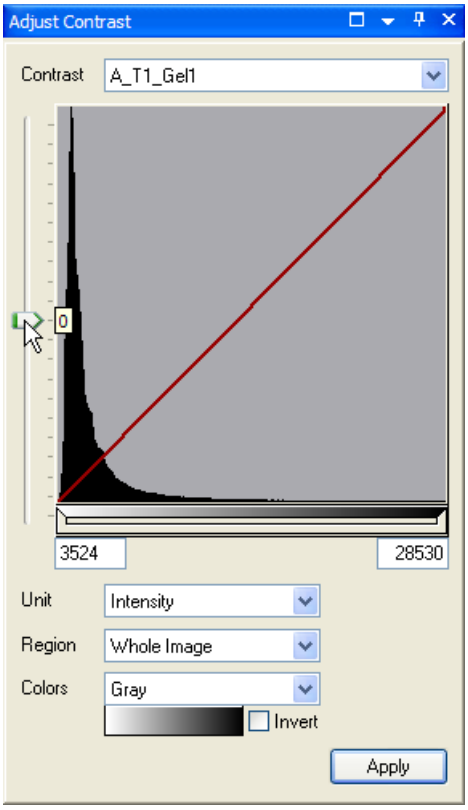


Figure 5-4. Adjust contrast function.

Change the default mapping

The software offers two complementary ways to change the default mapping function in order to improve the visual display of the gels (Figure 5-5):

- You can define the minimum and maximum gray levels (i.e., look at only the light or the dark regions in the images). Do this by decreasing the size of the gray level range that is to be remapped linearly to the screen gray levels. Move the left or right borders of the slider that is found below the histogram function. Once the size (interval) of the slider is decreased, you can also displace the interval to the left or right. Alternatively, you can type valid numbers in the boxes at the lower left and right corners of the histogram.

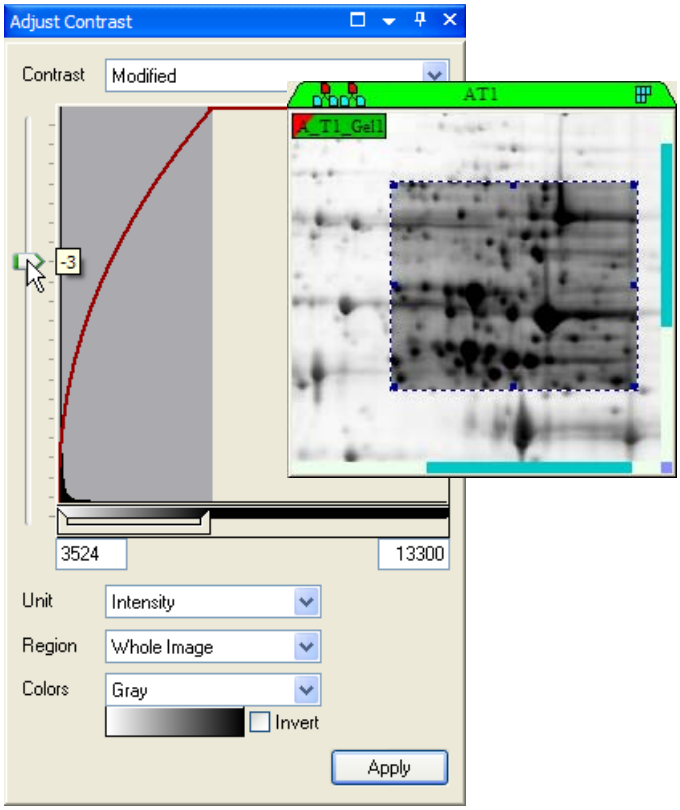


Figure 5-5. Adjustments to the gray level mapping for the selected image are immediately reflected in the preview region. In this example, the maximum gray level was set to 13300 by moving the right side of the slider to the left. All pixels with a gray level higher than 13300 appear as black. By setting the Bending slider to -3, the image becomes darker.

- You can additionally use a non-linear mapping function. The Bending parameter, i.e., slider to the left of the histogram, expands or compresses the contrast at the dark or light ends of the range. When the bending parameter is positive, the image is lighter. When the bending parameter is negative, the image is darker.

Contrast

Select the image for which you want to see the current minimum and maximum gray level settings. When you make changes to these settings, the list will display ***Modified*** to reflect this fact.

Unit

Two different units can be used for displaying the gray level minimum and maximum:

- ***Intensity*** uses the raw pixel values as displayed in all the reports. When a calibration is done, these correspond to the calibrated pixel values.
- ***%***: Chooses the scale as a percentage of the total gray level range in the histogram.

Region

You can display the histogram for the gray levels in the:

- ***Whole Image***. This is the default option.
- ***Selected Region***. The software only considers the gray levels that are present in the selected region. This option is practical in combination with the % unit and a relatively small region. In this case, you enter an adaptive mode that allows you to adjust to the local gray levels. The effect is a regional increase in contrast that is useful for viewing very faint spots.

Colors

The software offers various color palettes to visualize the intensities in your image. The Gray+Saturation palette is helpful when you want to visualize saturated spots or the background. It corresponds to the default Gray option, except for the maximum gray value (saturation) that is displayed in red, and the minimum value (background) that appears in blue. To decrease the stringency on what is considered saturation or background, you can modify the minimum and maximum gray levels.

Invert

You can inverse the gray levels by checking the ***Invert*** box.

5.3.2 3D view

Another way to examine the intensity variations in gel images is by looking at the three-dimensional view of gel regions (Figure 5-6). In this type of view, the X and Y axes represent the pI and MW values, whereas the pixel intensity is plotted along the third dimension (Z axis). The resulting image shows a peak for each protein spot, with a peak height that is proportional to the spot intensity. 3D views can be rotated in any direction to look at the interesting spots from all sides, thus facilitating spot editing or matching decisions.

The 3D View window can be displayed by choosing ***Reports > 3D View***. If spots are selected, the area around these spots is visualized for all images in the sheet (Figure 5-7). If only regions were defined on images in a sheet, these regions are shown in the 3D View (Figure 5-6). If both spots are selected and regions are defined, the area around the spots is shown by default but you can choose to display the regions.

The layout of the images in the 3D View reflects as much as possible the layout of the images in the sheet. Bold black lines separate images that are in different panes. You can restrict the number of images displayed

in the 3D View by setting their ***Visibility*** in the ***Display Options*** (see below).

> **CAUTION!** To ensure satisfactory performance, the number of images that can simultaneously be viewed in 3D view is limited to 24. If you selected more than 24 images, you will be able to choose the visible images from a list that contains information about the match sets or classes to which the images belong.

By default, 3D views can be rotated by dragging the mouse while holding down the left button. If your mouse has a scroll wheel, it can be used to zoom in or out on the view. You can change to one of the other image manipulation tools avaible in the ***Tools*** icon drop-down list (see below for more details). Please note that all views in the 3D View window are manipulated simultaneously.

Right-clicking is reserved for the contextual menu. It allows you to select the spot where your cursor is positioned and to quickly access one of the image manipulation tools described below.

The pixel gray levels are overlaid on 3D views; the top of the peaks are therefore always darker.

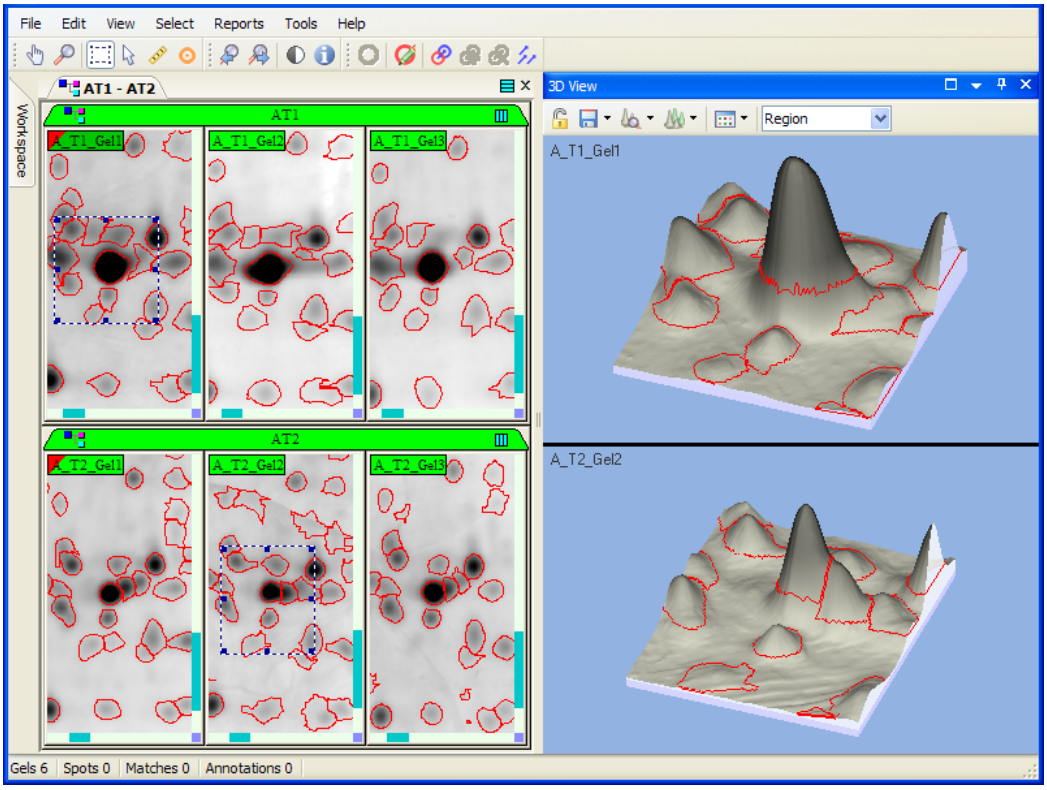


Figure 5-6. 3D views of the regions defined in A_T1_Gel1 and A_T2_Gel2.

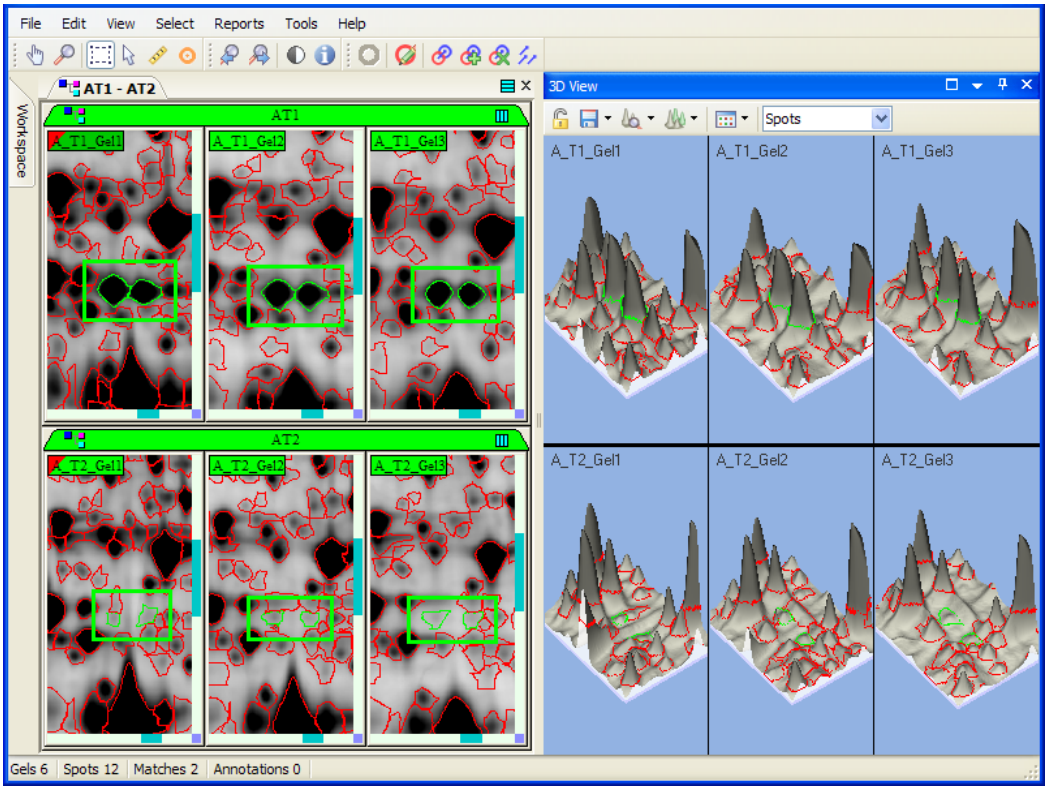


Figure 5-7. 3D views for the selected spots.

The following tools are available in the 3D View toolbar:

Tools

To manipulate 3D views with your left mouse button, you can switch to one of the following options:

- ***Rotation***: Rotate the images using your mouse.
- ***Zoom/Contrast***: Zoom in or out by dragging the mouse horizontally. Adjust the contrast, or height of the spots, by dragging the mouse vertically on the view.
- ***Translation***: Choose one of the suboptions (Z, Y, X, XY) to move the views along the desired axis.
- ***Auto Rotate***: Let the images rotate automatically around the Z axis so that you can view the spots from all sides.
- ***Show Default View***: Return to the original view.

Stack

- ***Stack***: Display the images one on top of the other, instead of using the default tiled view.
- ***Next/Previous***: Move to the next or previous image in the stack.
- ***Animate***: Switch automatically between the different images in the stack. This option is useful for visualizing the expression variations in a set of images.
- ***Transparent Mode***: Display one of the images (Reference) with a transparent surface. This option is useful for visualizing expression variations in a single, static view. To move one view with respect to the other, choose ***Translation*** in the ***Tools*** icon. Hold down the Ctrl key (gray) or the Shift key (green for the Reference) while dragging the view.
- ***Set Reference***: Select the image to be used as the Reference for the Transparent Mode.
- ***Transparency Settings***: Choose the color for the display of the Reference, and increase or decrease the transparency.

Display options

- ***Visibility***: Select the images to be displayed in the 3D View window.
- ***Spot Shape***: Set the way the spots are displayed in the 3D View (Crossed, Outlined, Filled, None).
- ***Display Options***: Display the X (pink), Y (purple) and Z (blue) axes, the coordinates of the point on which these axes are centered, and change the way the surfaces of the 3D Views are visualized (Grid, Wireframe, Smooth).
- ***Color Palette***: Combine the 3D View with one of the color palettes available.

5.3.3 Profile

It is sometimes difficult to judge whether spots should be split, are saturated or not, or have other problems such as so-called donut structures (low intensities in the center compared to the borders). It is important to identify such problems as they will lead to incorrect spot quantitation.

The Profile function, activated or deactivated by choosing ***View > Global > Show Profile*** can help in such cases. Red curves represent the intensity variations of the gel in the vertical and horizontal directions at the position of the mouse cursor (Figure 5-8). The Profile can clarify the intensity changes in a gel and assist in making editing decisions, without the need to open an additional window, as is the case with the ***3D View*** tool.

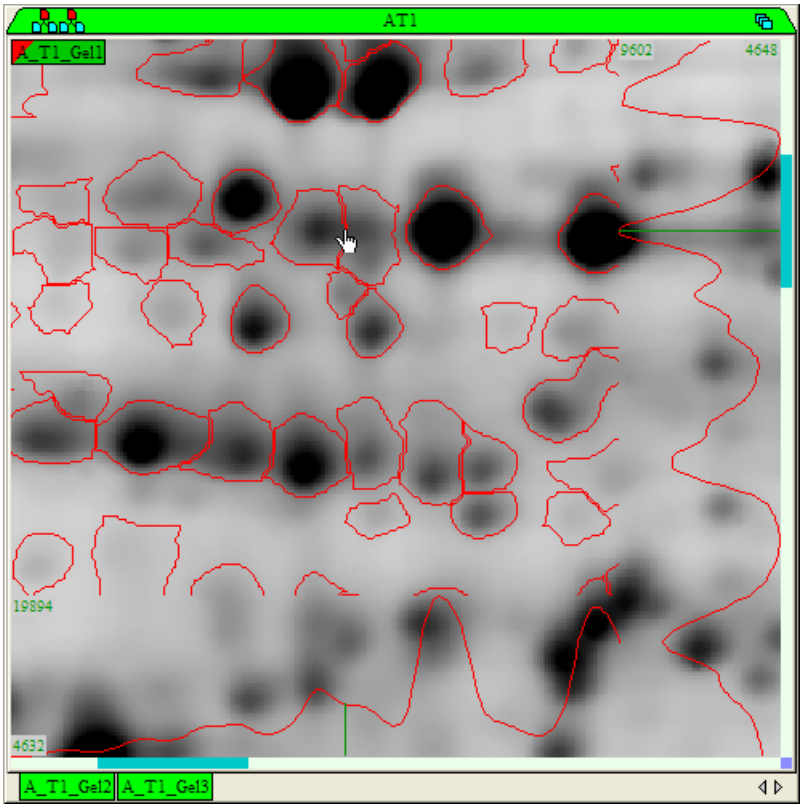


Figure 5-8. Profile feature. Red curves represent the intensity variations of the gel in the vertical (right) and horizontal (bottom) directions at the position of the mouse cursor. Green lines indicate the exact position of the cursor, whereas the numbers indicate the minimum and maximum gray levels in a specific profile view.

5.3.4 Cursor information

At any given time, the Cursor Information window can be used to display information on pixels such as the pixel intensity and the X and Y coordinates expressed in pixels, in pI and MW units (if available), and in cm or inches. Note that if spots are detected, the Cursor Information window also displays spot information.

The Cursor Information window is availabe by choosing ***View > Global > Cursor Information***, or by clicking the corresponding icon in the Display toolbar. Just place the cursor over the pixel for which you want to display information.

5.4 Visually compare images

When working in MatchSet or Classes sheets, the software provides different tools to visually compare images to the sheet reference.

5.4.1 Sheet reference

You can set the sheet reference by choosing ***View > Sheet > Set Reference***. All other images in the sheet are compared to this reference image for the options in the ***View > Sheet*** menu (Align Images, Show Dual Color,Spot Overlap) .

5.4.2 Align images

The Align Images feature facilitates the visual comparison of images that demonstrate large variations in protein migration. Choose ***View > Sheet > Align Images*** to activate it. It is especially helpful when the images are in Stacked mode. It warps (Figure 5-9) the images in the sheet so that they are better superimposed with the sheet reference and therefore with each other. This allows easy identification of corresponding spots.

To align images, the software needs to know which positions in the different images correspond to each other, that is, represent the same protein form. This is done by defining landmarks. The alignment algorithm then deforms the images to superimpose the landmarks.

> **CAUTION!** As opposed to matching, landmarks do not need to be linked with spots to carry out image alignment. Therefore, no spots need to be detected.

To align images in a sheet:

1. Set the desired image as the sheet reference, with ***View > Sheet > Set Reference***.
2. Select the ***Landmark*** tool and define a few landmarks, bearing in mind the rules listed below.
3. Choose ***View > Sheet > Align Images***.
4. If parts of your images are still not sufficiently aligned, you can add extra landmarks. The alignment is automatically updated.
5. To view the original images, choose ***View > Sheet > Align Images***. The original images replace the aligned ones..

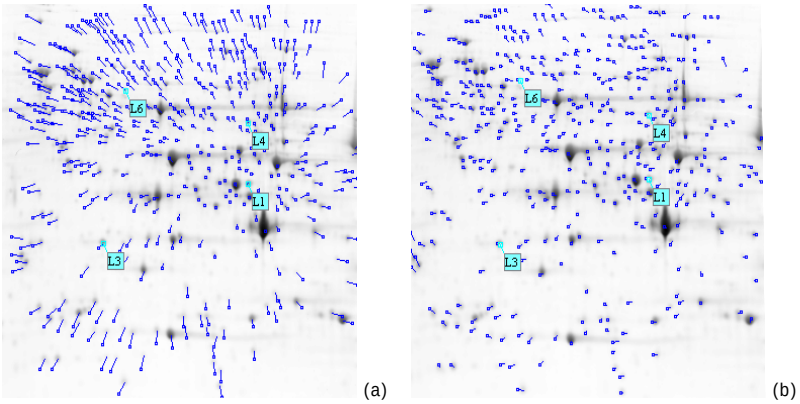


Figure 5-9. Image (a) before and (b) after alignment. The match vectors and landmarks are displayed.

Note that the addition of suspect or badly positioned landmarks can worsen the alignment results. The following rules must be considered when defining landmarks:

- The number of landmarks should be kept to a minimum. There is no point in putting a landmark on each spot.
- Landmarks should be well distributed over the whole images (covering both the X and Y directions). To correct for local distortions, it is recommended to define landmarks around the distorted regions rather than within those regions.
- Landmarks should only be defined on spots that clearly represent the same protein form. Protein variants definitely should not be used as landmarks.
- Landmarks should be placed on small, sharp spots (of similar area), rather than on large diffuse ones (which may differ considerably in size) because in the latter case the error in the position is much more substantial.
- Landmarks should be added gradually so that you can monitor their individual influence on the alignment (automatically updated after each landmark addition). Immediately remove landmarks that decrease the alignment quality.
- When a spot is missing on a gel (sometimes happens to border spots), you should not put a landmark (i.e., validate a landmark) in a hypothetical spot position. Missing landmarks are not an issue.

The Align Images feature is a purely visual tool. It is not used in, and will not improve, the matching process. The only reason to align your images is to ease their visual comparison (possibly in combination with the Dual Color mode). Alternatively, double-click in an image (with the ***Move*** tool selected) to locally superimpose the different images:

- If the images are matched, the image positions are synchronized based on the surrounding matches.
- If the images are not matched but have landmarks, the image positions are synchronized based on a simple interpolation between the two nearest landmarks.
- If no matches or landmarks are present, the image positions are synchronized based on the same image location (X,Y coordinates).

5.4.3 Show dual color

Choose ***View > Sheet > Show Dual Color*** to display each of the images (the sheet reference and the current image) in one of two colors: red and cyan (Figure 5-10). Remember that you can select the sheet reference by choosing ***View > Sheet > Set Reference***. When the pixel colors of the two superimposed gels are added:

- Overlapping spots appear as shades of gray.
- Cyan spots are present only in the current gel.
- Red spots are present only in the sheet reference.
- Halos of cyan or red around dark spots indicate that the protein is over or under expressed, respectively, compared to the sheet reference.

The less color you see in the dual color mode, the more similar the gels. Of course, this is only true if the gels are correctly aligned (see above) and superimposed.

CAUTION! It is recommended to perform any operations related to gel alignment (especially the definition of landmarks), before entering the Dual Color mode to avoid a slow down due to the recalculation of the overlaid images.

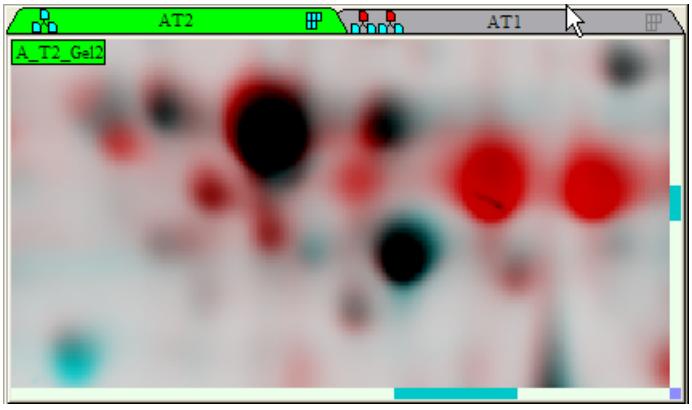


Figure 5-10. Dual color view.

5.4.4 Spots overlapped

Chooose ***View > Sheet > Spot Overlap*** once spots have been detected and the visible spots on the sheet reference will be shown in blue on the image (Figure 5-11). Thus, you can easily compare the position and size of the red spots in the current gels with the blue spots on the sheet reference (shown in Crossed or Outlined mode).

You can change the default color to be used for overlapped spots by going to the ***Display*** tab in ***Tools > Options***, and clicking the ***Overlapped*** box. Remember that you can change the sheet reference by choosing ***View > Sheet > Set Reference***.

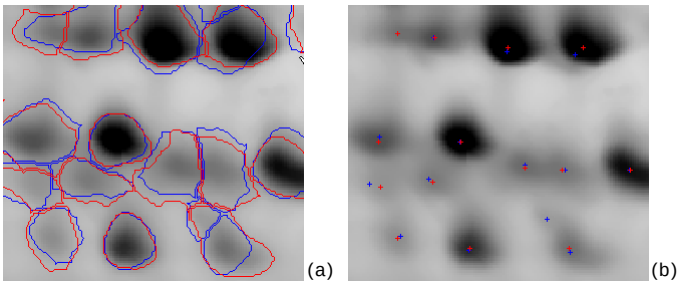


Figure 5-11. Spots overlapped with (a) outlined and (b) crossed spot shapes.

5.5 Grid lines

5.5.1 Display grid lines

Choose ***View > Global > Grid Lines > Show*** to display grid lines over your images (Figure 5-12). Grid lines can be used to evaluate distances between spots, in terms of pixel coordinates, pI/MW units, or real world units (cm or inch). Grid lines are also a helpful way to visualize deformations in aligned gels because the grid lines are warped in the same way as the image.

5.5.2 Edit grid lines

Choose ***View > Global > Grid Lines > Edit*** to change the grid properties. The software trys to partition the visible area into the ***Number of subdivisions*** entered by the user (Figure 5-12). The graduations can be attached to the ***Gel*** or the ***Screen***. This means that the software divides the gel width/height or the visible screen area by the number of subdivisions. The ***Coordinate Units*** can be Centimeters, Inches, Pixels, or pI/MW units, provided data is available in the annotation category pI_MW. The pI/MW grid can also be displayed over gels that do not

contain this information, but that were matched to a gel having pI and MW values.

.

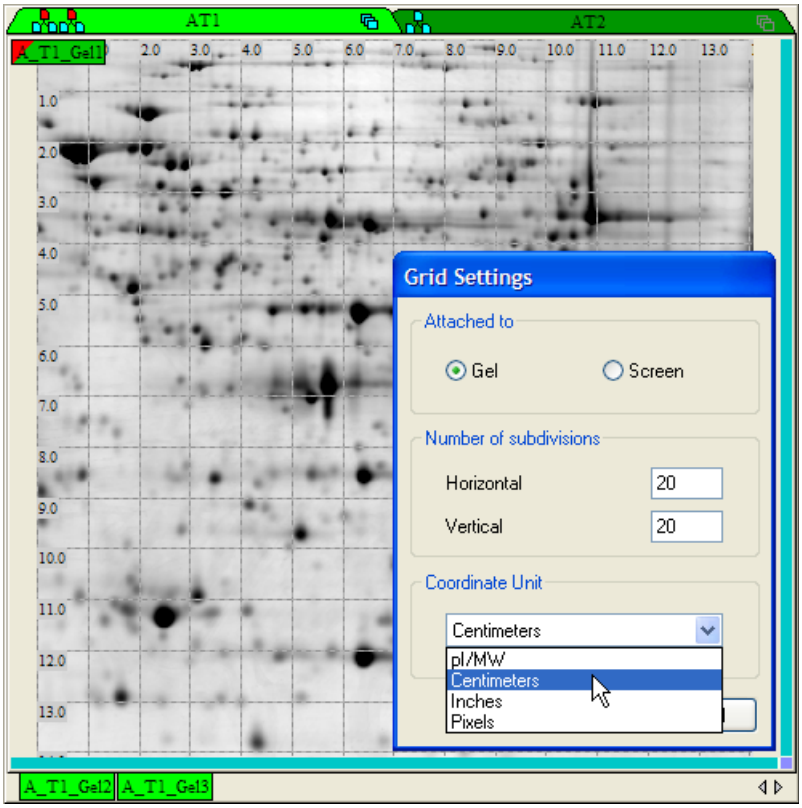


Figure 5-12. Grid lines in cm attached to the gel image.

5.6 Gel reports

5.6.1 Gel table

Choose ***Reports > Gel Table*** to display a table (Figure 5-13) with summarized information about the images in the current sheet:

- File name, ID, path, size, modification and creation dates.
- Image height and width expressed in pixels (***Rows*** and ***Columns***), pixel size (***PixSize***).
- Minimum and maximum gray levels before (***MinGray*** and ***MaxGray***) and after calibration (***MinValue*** and ***MaxValue***).
- Calibration information (***Calibration Formula***, ***Unit***, ***Name***, ***Creator*** and ***Date***).

- Number of spots and annotations.
- Staining method and user-defined gel descriptions.
- ***MatchSet*** to which the image belongs and the ***Class*** to which it was assigned.

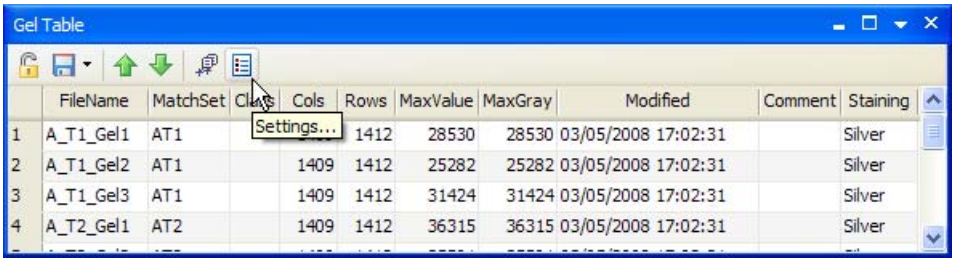


Figure 5-13. Gel Table.

You can customize the Gel Table to display only the columns of interest. Four predefined report templates are already available from the ***Load*** icon in the ***Settings*** of the Gel Table toolbar:

- ***Properties***: Shows various properties of the gel images: File Name, image height and width in pixels, maximum gray levels before and after calibration, Modification Date, as well as the MatchSet and Class to which it belongs.
- ***Files***: Shows information on the image files: File Name, ID, Path, Size, Creation and Modification Dates, as well as the MatchSet and Class to which they belong.
- ***Descriptions***: Shows the user-defined gel descriptions.
- ***Calibration***: Provides calibration-related information: File Name, ID and Path, as well as Calibration Formula, Name, Creator and Date.

Click the ***Add Description*** icon in the table toolbar to add or edit the Comment, Staining or other user-defined gel description fields for selected gels.

5.7 Save, export and print images

5.7.1 Save

The software automatically saves your images and all associated data as part of the project when you close a sheet or exit the software. You can also save your work by choosing ***File > Save***.

5.7.2 Export

Rather than saving your gel images in the Melanie file format, you may want to export them to a different file format (TIFF, BMP or PNG). The gel images are exported as 8-bit, flat, rasterized images without any

structure. This means that gel components such as spots and annotations are saved exactly as they appear on the screen, but are no longer recognizable as Melanie objects and therefore become part of the image. Consequently, exported gel images should only be used for presentation purposes and not for further analysis with any software package.

Please note that you can adapt the size of the exported image by zooming in or out. If a region is selected on the image, you are given the choice to export the entire image or the selected region only.

Alternatively, you can export images to the clipboard for direct pasting into another software. The procedure is very similar to exporting images to files. However, you can only export one image at a time to the clipboard.

Finally, you can also export a view of the current sheet.

The following export options are available in the ***File > Export*** menu:

- ***Image to Clipboard***
- ***Image to File***
- ***Sheet to Clipboard***
- ***Sheet to File***

5.7.3 Print images and sheets

Print options

The software provides various printing options in the ***File > Print*** menu:

- ***Images***: Print selected images or image areas (if defined with the ***Region*** tool). This option prints one image per page.
- ***Sheet***: Print the current sheet.

Whatever your choice, the image is printed as it is displayed on the screen retaining objects and properties such as spots, annotations, contrast mapping and pseudo colors, alignment, zoom, grid, etc.

> **CAUTION!** With a zoom factor of 1, the printed gel image takes the full paper width. You can adapt the zoom factor to decrease the size of the printed image.

Page Setup

You can change print parameters such as printer name, paper size, paper orientation, etc. Choose ***File > Page Setup***. This command opens the standard print window where printer-related settings can be modified.

6 Spots

6.1 Introduction

Once gels have been added to a project and you have taken a good look at them, you are ready to detect spots. A spot delineates a small region in the gel where protein is present. This shape is automatically differentiated by a spot detection algorithm and quantified; its intensity, area and volume are computed.

There are two different spot detection algorithms implemented:

- Non-DIGE images are detected with the Melanie algorithm.
- DIGE images are co-detected with GE Healthcare's DeCyder 2D algorithm.

6.2 Detect spots in non-DIGE gels

6.2.1 Procedure

The Melanie spot detection algorithm is optimized to give relevant biological results with minimum user interaction. You can preview spot detection and fine-tune just a few parameters before automatically locating all the spots in your image.

To detect spots automatically:

1 Display the gels to be detected by selecting a match set in the Workspace. Right-click and select ***Display*** in the contextual menu.

2 Choose ***View > Spots > Outlined*** to visualize the spot borders.

3 Click the ***Region*** tool. Draw preview regions on one or more gels. Note that you can still draw, resize or move regions while setting the detection parameters.

4 Select the gel images for spot detection.

5 Choose ***Edit > Spots > Detect***.

6 The Detect Spots window appears on-screen and the spots in the preview regions of the selected images are detected with the default parameters. If you do not want to recalculate the spots in the preview regions for each parameter change, turn the ***Auto Preview*** option off. To manually refresh the preview regions, simply click the ***Preview*** button.

7 Adjust the detection parameters (Figure 6-1). In particular optimize ***Smooth*** to detect all real spots and split the overlapping ones. Subsequently, filter out the noise by changing the ***Saliency*** and ***Min***

Area values. See below for more details on spot detection parameters.

8 When you are satisfied with the preview, click ***OK*** to detect all spots in the selected gels using the specified parameter values. Note that you can still change your gel selection at this point.

9 The spot shapes are displayed on the images.

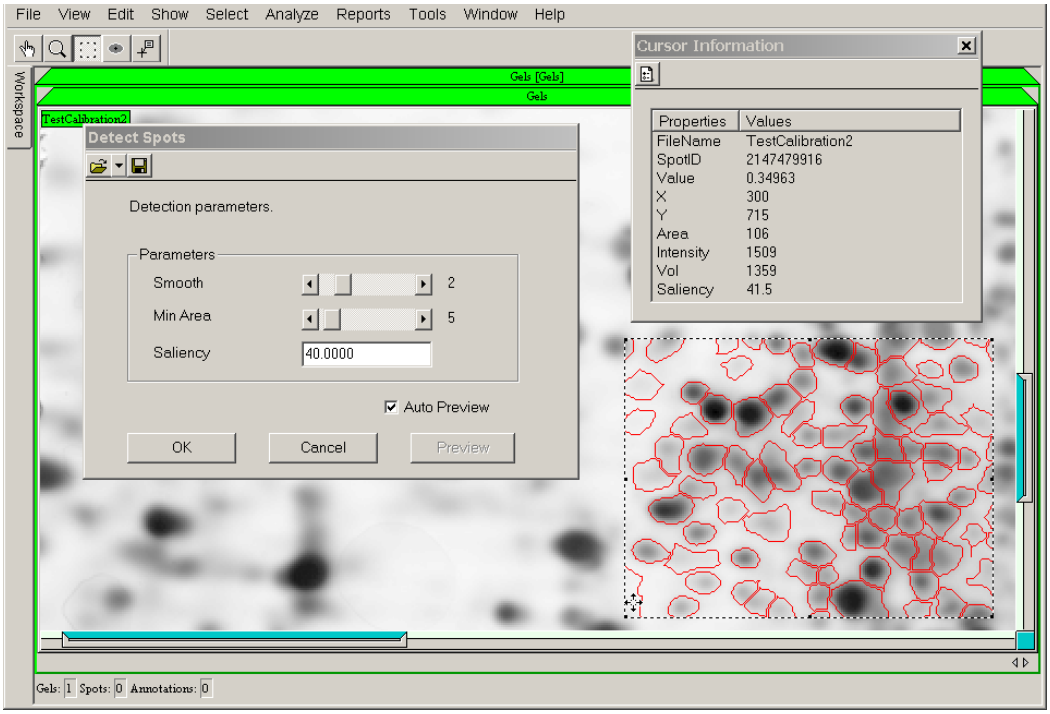


Figure 6-1. Adjusting spot detection parameters in real time.

6.2.2 Spot detection parameters

Spot detection parameters are best adjusted in the following order:

- **Smooth**: Set this parameter first. It fixes the number of times Melanie smooths the image before detecting spots, using a smooth-by-diffusion algorithm. The Smooth parameter should be optimized to detect all real spots and split as many overlapping spots as possible without being concerned about noise spots (these can be filtered out with the Saliency and Min Area parameters).
- ***Saliency***: This parameter is a measure based on the spot curvature. It indicates how far a spot stands out with respect to its environment. Real spots generally have high saliency values whereas artifacts and background noise have small saliencies. Although the Saliency is an efficient quantity for filtering spots, it is also highly dependent on the images (e.g., image resolution and

depth). Some gels need a saliency value of 10 for correct filtering. Others may necessitate a value of 5000. To estimate the saliency range to use with your images, you can display the Cursor Information window or Spot Table and look at the saliency value given for a spot that you would like to suppress. Enter this value in the Saliency field in the Detect Spots window. The spot detection algorithm then discards all spots with saliencies smaller than the specified threshold.

- ***Min Area***: After setting an appropriate Saliency to filter out all noise spots, there may still be noise in your gel that cannot be eliminated without suppressing real spots. This often happens with dust particles that consist of a few very dark pixels. Get rid of these artifacts by using the Min Area parameter. It eliminates spots that have an area smaller than the specified threshold (expressed in number of pixels).

6.2.3 Spot quantification

The software automatically computes the amount of protein present in each spot. Figure 6-2 illustrates the principles of spot quantification in the Melanie algorithm. Measuring the protein quantification values in

this way has the advantage of being more robust and reproducible when calculating protein expression variations (relative quantification).

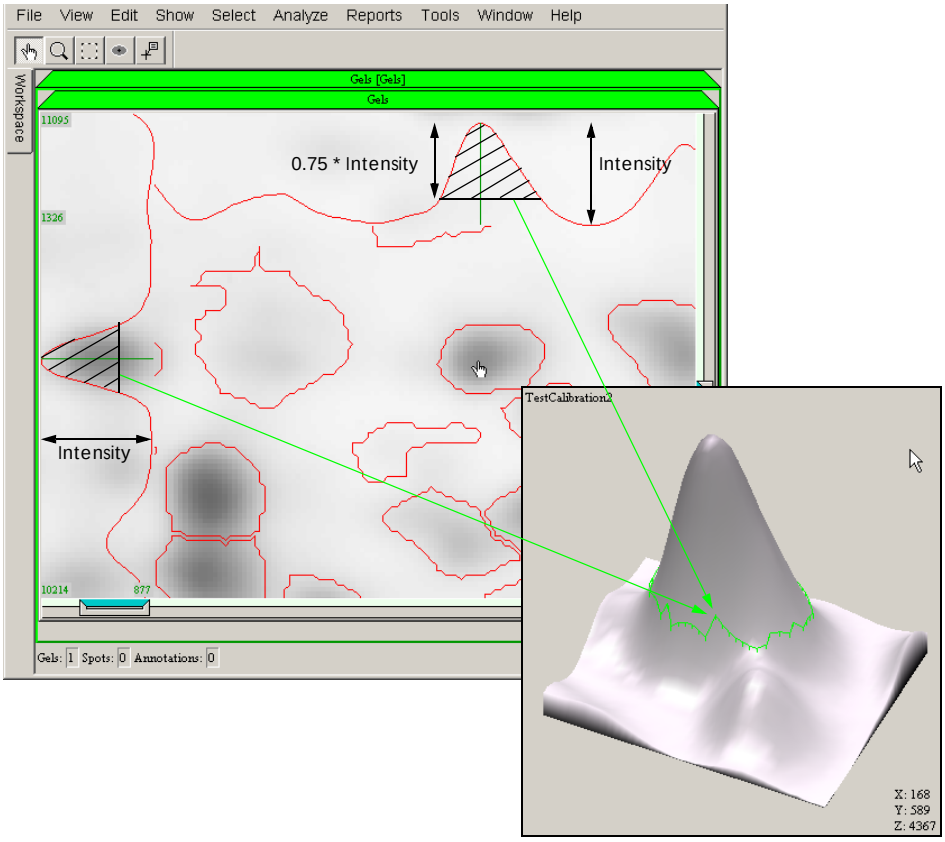


Figure 6-2. Spot quantification. The 3D View in Melanie reflects the spot shape and volume of what will effectively be quantified. The spot outline corresponds to the area at 75% of the spot height when measured from the peak.

- **Intensity**: The software first calculates the intensity of a spot. The intensity is based on the highest calibrated pixel intensities in the spot from which the background has been withdrawn. The background is defined as the minimum pixel value in the spot neighborhood.
- **Area**: The area of a spot is not determined at the spot base because the base is often arbitrary and difficult to determine. It is calculated at an intermediary height of the spot. More precisely, Melanie computes the area at 75% of the spot intensity, as measured from the peak of the spot. The spot outlines displayed in

Melanie exactly encircle this computed spot area (expressed in mm^2).

- **Vol**: The volume of a spot is calculated as the volume above the spot outline, which is situated at 75% of the spot height (as measured from the peak of the spot). In Figure 6-2, the measured volume of the spot is hatched. Please note that the volume values, like the intensities, depend on pixel intensity calibration.
- **%Vol**: The relative volume of a spot is calculated as indicated below. It is a normalized value that remains relatively independent of variations due to protein loading and staining by considering the total volume over all the spots in the image. This means that in an image where, globally, the spots are darker than in another image, the majority of spot volumes is higher. However, the bulk of %Vol should be similar to those in the compared image, at least for gels with similar spot patterns.

$$\%Vol = \frac{Vol}{\sum_{S=1}^{n} Vol_S} \times 100$$

where Vol_S is the volume of spot S in a gel containing n

CAUTION! When you look at a detected spot in the 3D View, you will notice that the borders are not localized at the base of the spot, especially for intense spots. This is normal because the spot contours displayed effectively reflect the quantified parts of the spot, and they do not correspond to the 'whole' spot, which is difficult to define.

6.3 Co-detect spots in DIGE gels

The co-detection algorithm is designed to simultaneously process one, two or three images derived from a single gel.

- **Single detection**: one image.
- **Double detection**: two images.
- **Triple detection**: three images.

Single detection is performed on images of fluorescently post-stained gels used for picking, a case where there is a single image associated with the gel.

Double and triple detection takes advantage of the inherent co-migration benefits of the CyDye™ DIGE Fluor dyes. A set of co-run images are merged together thereby incorporating all spot features in a single image. Spot detection and spot boundary definition is then performed using pixel data from all the individual raw images and the merged image. The resultant spot map is overlaid back onto the original

image files. Since the spot boundaries and the detection areas are identical for all images, the spots are effectively already matched. This process results in highly accurate volume ratio calculations.

6.3.1 Procedure

To perform spot detection on DIGE images:

1 Display the gels to be detected by selecting a match set in the Workspace. Right-click and select ***Display*** in the contextual menu.

2 If desired, select a subset of gels to be detected.

3 Choose ***Edit > Spots > Detect***.

4 In the DIGE Spot Detection window, enter an estimation (see below) of the ***Number of Spots*** present in the images. Click ***OK***.

6.3.2 Spot detection parameter

When detecting DIGE images, you must enter an estimation of the Number of Spots present in the images. It is recommended that this value be overestimated to compensate for the detection of non-proteinaceous spots on the image, e.g., dust particles which can subsequently be excluded from the analysis using spot filtering.

If all the spots are not identified, the spot detection process can be repeated with a higher number of estimated spots.

For example, for a mammalian lysate run on an 24 cm pH 4-7 Immobiline™ DryStrip and a large format gel, such as the Ettan™ DALT Gel (20 cm x 26 cm), a value of 2500 for the estimated Number of Spots should be satisfactory.

6.3.3 Spot quantification

Volume, area, intensity, slope, and volume ratio for individual spots are automatically calculated and included in the Spot Table and Cursor Information window.

- ***Vol***: Spot volumes (sum of pixel intensities within the spot boundary) are always expressed with background subtracted. Background is subtracted on a spot specific basis by excluding the lowest 10th percentile pixel value on the spot boundary from all other pixel values within the spot boundary. The spot volume is the sum of these corrected values.
- ***Vol ratio***: Volume ratios (volume of current image spot/volume of DIGE reference image spot) indicate the change in spot volume between two images.

CAUTION! When using single detection the volume ratio value is 1.0 for all spots since there is no second image.

6.3.4 Exclude spots

To exclude small, non-proteinaceous spots on DIGE images, it is recommended to filter the spots based on the Volume. More precisely, spots that have a maximum volume in the two or three images that is lower than a given threshold can be deleted.

To exclude DIGE spots based on maximum volume:

1 Choose ***Reports > Spot Table***.

2 Click the ***Select by Value*** icon in the toolbar of the table.

3 Select ***Volume*** in the displayed list. Make sure the **>=** box is checked and enter a value to be used as the cutoff for the volume. Deselect the **<=** box and click ***OK*** (Figure 6-3).

4 All spots with a volume higher than the given threshold are selected, together with their matched spots (which may have volumes that are smaller than the threshold). These are the spots that are kept.

5 Choose ***Select > Spots > Inverse Selection***.

6 The current selection includes all spots for which the maximum volume in any of the two or three DIGE images is lower than the given threshold.

7 Choose ***Edit > Spots > Delete*** to permanently delete the selected spots from your DIGE gels.

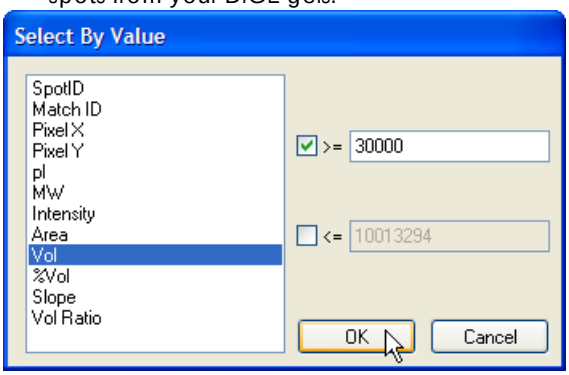


Figure 6-3. Select by Value can be used to filter spots based on their volume.

Please note that instead of deleting spots, you can exclude them from the analysis by disabling them.

6.4 Select spots

6.4.1 Select

Spots can be selected with the ***Select*** tool. Once selected, they are highlighted in green (Figure 6-3), unless the default spot colors were modified. Note that all matched spots are selected as well.

If an annotation is attached to a spot, the annotation is also selected. Similarly, if you select an annotation or label with the ***Select*** tool, the linked spot is also selected.

To select more than one spot, select the first one and then hold down the Shift key while clicking on additional spots.

To select all spots in a region, place the cursor at the top left position of the desired region, hold down the left mouse button, and then drag the cursor to the bottom right position. All spots in the designated region are selected.

To deselect all spots, click in the gel (not on a spot).

Surrounding box

By default, spot selections are surrounded by green boxes. This makes it is easier to localize selected spots, especially when working at low zoom factors. To deactivate this option, choose ***Tools > Options***, and uncheck the corresponding box in the ***Display*** tab.

Alternatives

There are other ways to select spots:

- Choose a command in the Select menu.
- Select spots in reports.

6.4.2 Spot sets

It is possible to focus your analysis on particular spots by creating and saving spot sets for later selection or combination.

Create

Select spots you want to include in a spot set, either manually or by selection in a report. Then choose ***Edit > Spot Sets > Create***, enter a name for the new spot set and click ***OK***.

Spot sets can be visualized as columns in various reports (Figure 6-4). If they are not displayed by default, you can add them by checking the corresponding box in the ***Settings*** of the report window (only for tabular reports). Once the column is displayed, you will see a checked box for spots that belong to the set, or an empty box for spots that do not belong to the set. Click in a box to change its state. If several spots are

selected, you can change the state by clicking in one box while holding down the Shift key.

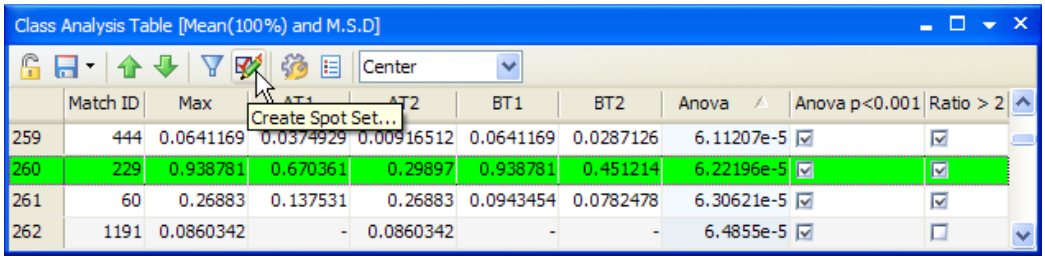


Figure 6-4. Spot sets Anova p<0.001 and Ratio > 2 in a Class Analysis Table. New spot sets can be created by clicking on the Create Spot Set icon.

Spot sets can also be created by clicking the ***Create Spot Set*** icon in a report toolbar. All currently selected spots are automatically included in the newly created spot set.

Delete

Delete a spot set by choosing ***Edit > Spot Sets > Delete*** and selecting the spot set(s) to be deleted.

Combine

You can combine two spot sets using logical operators, in order to create a new spot set or change the current selection (Figure 6-5). Four operators are available:

- **And**: Keeps spots that belong to both spot sets.
- **Or**: Keeps spots that belong to either one or both spot sets.
- **Not equal**: Keeps spots that belong to only one of the two spot sets.
- **Exclude**: Keeps spots that belong to the first spot set and do not belong to the second spot set.

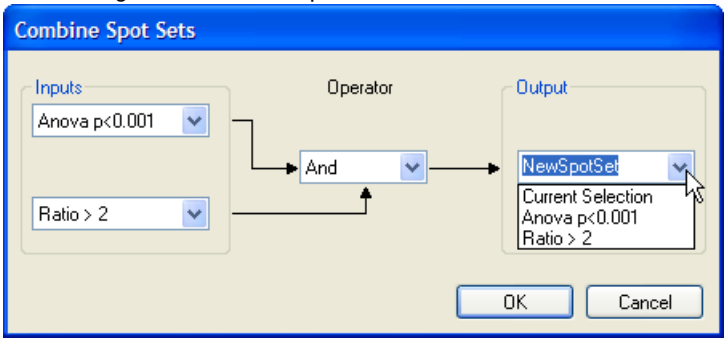


Figure 6-5. Combine Spot Sets window.

To combine two spot sets:

1 Choose ***Edit > Spot Sets > Combine***.

2 From the ***Inputs*** drop-down lists, select the spot sets to be combined. The current selection can also be used as input.

3 Specify the operator (And, Or, Not Equal or Exclude).

4 Enter a new name for the resulting spot set. Alternatively, the output can be immediately reflected in the current selection. Click ***OK***.

Select
To select spot sets, choose ***Select > Spot Sets***.

6.4.3 Enabled spots

By default, all spots are enabled and therefore represented in the reports. To exclude a specific subset of spots, you can disable protein spots that are not of interest, or specifically define a set of spots to be enabled. Only enabled spots appear in reports.

The options related to the creation, saving or loading of sets of enabled spots can be chosen from the ***Edit > Enabled Spots*** menu:

Set
Select the spots you want to focus your analysis on and choose this option to enable the selected spots. After deselection, excluded spots are disabled and appear in yellow.

Add
Use this option to add selected spots to the current set of enabled spots.

Remove
Use this option to remove selected spots from the current set of enabled spots.

Save
Use this option to save the currently enabled spots as a new spot set. Enter a name and click ***OK***.

Load
Use this option to enable spots belonging to an existing spot set.

Select
To select the enabled spots, choose ***Select > All Enabled Spots***.

6.5 Display spots

6.5.1 Spot shape

Once spots are detected, you can choose how to display their shapes (outlined, crossed, filled, outline/filled) on the gels from the ***View > Spots*** menu.

6.5.2 Spot color

By default, enabled spots are displayed in red, disabled spots in yellow, selected spots in green and overlapped spots in blue. You can change these default colors in the ***Display*** tab in the Options window (accessible by choosing ***Tools > Options***). Click the colored box you want to change and the Color window opens. Choose the preferred color from the spectrum and click ***OK***.

6.5.3 Spot ID

You can display Spot IDs for selected spots with ***View : Spots : Show ID***. To hide all Spot IDs again, choose ***View : Spots : Hide All ID***.

6.6 Edit spots

CAUTION! Except for deleting spots, spot editing is not allowed on DIGE gels.

Quantitative protein data, and in particular the spot volume, are highly dependent on an optimal and reproducible definition of the spot borders and a correct splitting of partially overlapped spots. To guarantee reproducibility of quantitative work it is therefore recommended to create spots by using the automatic spot detection algorithm in the software and to avoid manual editing as much as possible.

However, spot detection differences can still occur. In particular, some spots are differently split in gels to be compared. The software offers the following solutions to deal with detection variations between gels without calling for spot editing:

- Exploit the capability to create multiple matches. In practice, this means that you can match “composite spots” that are treated as unique entities in the quantitation.
- Exploit the capability to propagate all or selected spots from one image to the other images.

CAUTION! Both solutions require prior matching. Therefore, before doing any spot editing, first match your images and only then consider to use one of the options described below.

6.6.1 Manual editing

You must enter the special spot-editing mode to manually edit spots. Choose ***Edit > Spots > Edit Enabled***, or click the corresponding icon in

the Detect And Match Spots toolbar. The Edit Spots toolbar displays. Selecting the Edit Enabled option again disables spot editing.

> **CAUTION!** For edited spots, the Saliency value becomes zero. This can be used to quickly check which spots have been edited.

The following spot editing tools are available:

Create spots
Click this icon and draw the outline of the new spot. Alternatively, double-click the desired location in your image, and set the disc radius for the circular spot to be drawn in the ***Create Spot*** window.

Delete spots
Click this icon, select the spot to be deleted, and confirm.

Split spots
Click this icon, select a spot to be split, and draw a line through the spot at the position where the separation should occur. Make sure you start and finish outside the spot.

Merge spots
Click this icon, select two or more spots to be merged, and draw a trajectory through the selected spots by making sure to start and finish in the same spot.

Grow spots
Click this icon, select a spot to be grown, and outline the area you would like to add by making sure to start and finish within the selected spot.

Shrink spots
Click this icon, select a spot to be shrunk, and outline the area you would like to suppress by making sure to start and finish outside the selected spot.

6.6.2 Composite spots

As shown in Figure 6-6, the software allows you to match several spots in one gel with multiple spots in other gels. In the figure, the three selected spots in gel A_T1_Gel1 are matched to the single green spot in A_T2_Gel1 and the two selected spots in B_T1_Gel1, and so on.

Once the match has been effectively created, the three spots in A_T1_Gel1, for example, are treated as a single entity in the quantification. The quantification value for A_T1_Gel1 displayed in the different reports is the one obtained after combination of the values from the three individual spots.

This is an efficient solution for dealing with spot detection differences without subjective and time-consuming spot editing. When selecting a spot on a gel, any matched spots on the other images are automatically selected as well. As a result, defining a multiple match is

very easy and quick. You just need to select all spots to be matched and click the ***Add Match*** icon in the Detect And Match Spots toolbar.

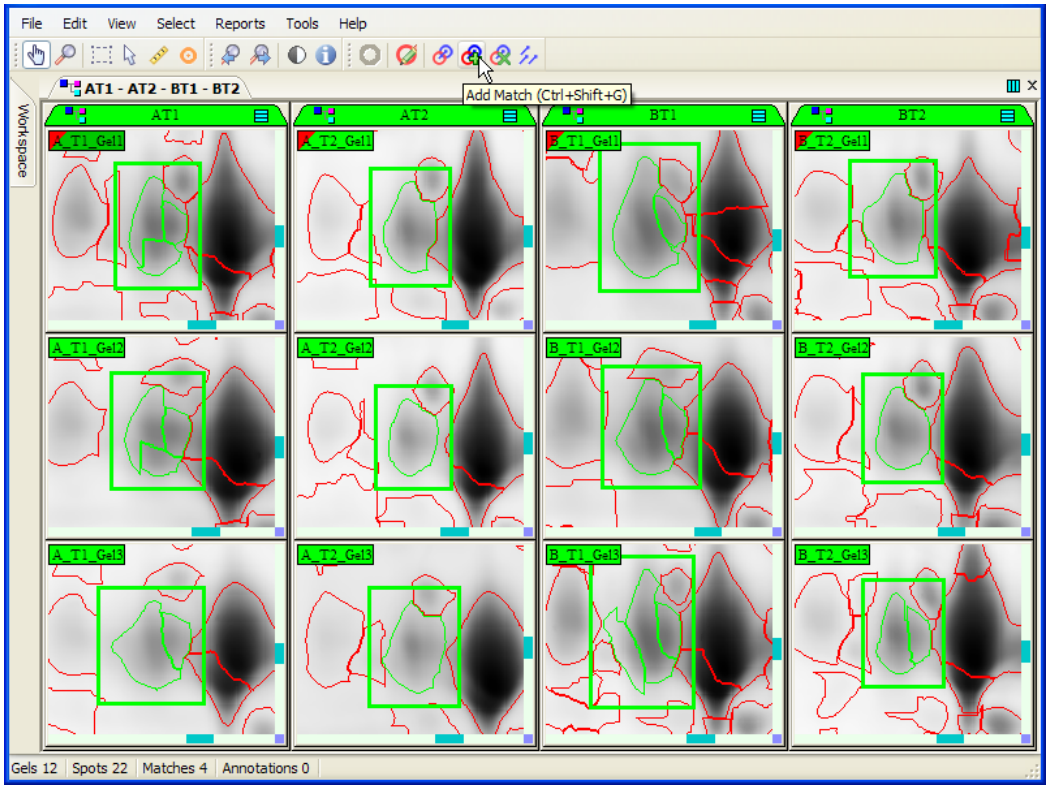


Figure 6-6. Composite spots can be defined through multiple matches

6.6.3 Propagate spots

You can propagate all or selected spots from one image to the other matched images (Figure 6-7). This allows you to quantify identical areas on all gels.

- **For matched spots**: the spot in the destination image is replaced with the shape of the spot in the source image.
- **For non-matched spots**: the spot from the source image is copied to the equivalent location in the destination image. This position is extrapolated from the surrounding match vectors.

Matches are automatically created between the original and the propagated spots.

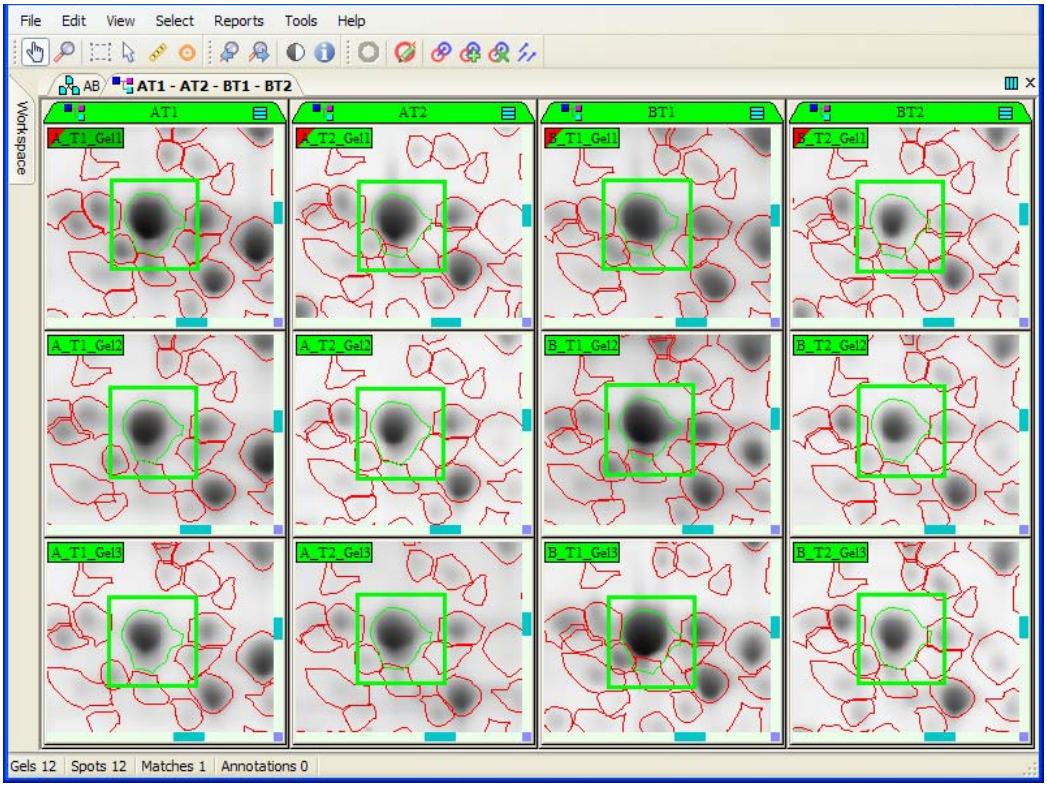


Figure 6-7. All spots from gel A_T1_Gel1 have been propagated to the other images.

To propagate spots from one matched image to another:

1 Select spots on one image.

2 Choose ***Edit > Spots > Propagate***.

3 Select one or more images you want to copy the spots to.

4 The new spots are added to the selected images.

6.7 MW and pI calibration

If you have a gel with pI/MW standards, the software can compute approximate pI and MW values for all the spots/pixels in this image, as well as any other images matched to it.

The principle is rather simple. You just have to define pI_MW annotations for a certain number of spots/pixels in the gel. The calculated pI and MW values for all spots in this gel and any matched gels are

automatically available in the Spot Report or Cursor Information window (see below). In addition, pI and MW grid lines can be displayed over the images.

To define pI_MW annotations on an image:

1 Select the image for which you know the pI and MW values for several protein spots. Spots may or may not have already been detected in this image.

2 Click ***Select*** in the toolbar.

3 Double-click on a spot (pixel) for which you know the pI and/or MW values.

4 Select the ***pI_MW*** category in the Create Annotation by Click window.

5 Enter the known pI and MW values, respectively, separated by a space. Replacing one of the values with -1 means that no value is set.

6 Do this for a sufficient number of protein spots that are distributed over the whole image area. Obviously, the more spots and annotations, the better the approximated pI and MW values will be.

Melanie does the following to calculate approximate pI and MW values. In the case of pI, it looks up the two closest annotations to the left and to the right of the spot for which the pI will be determined and then interpolates between these two points. Since Melanie does not have any information about the experimental (possibly non-linear) pI scale, the calculated values are only approximate. In the case of MW of the spots, the procedure is similar, except that Melanie searches for the closest spots above and below the spot for which the MW will be determined and it makes a logarithmic interpolation.

Extrapolating pI and MW values is more complicated. For example, if the pI of a spot on the extreme right side of your gel is to be determined, the software looks for the two closest spots to the left of the spot in question. If these two spots are sufficiently distant from each other (in order to decrease the error), the value for the spot in question can then be extrapolated.

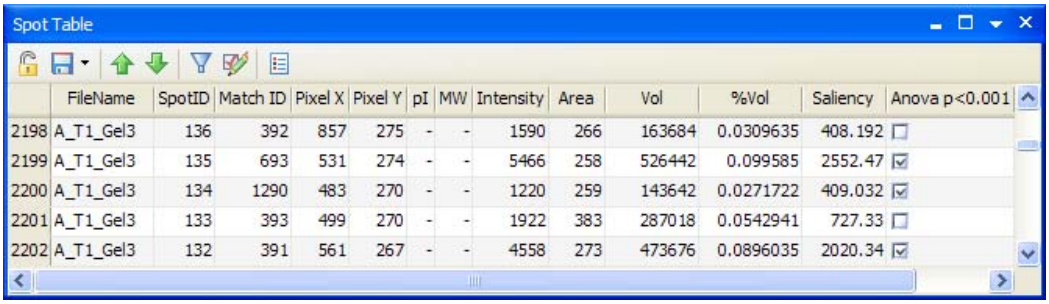
Normally, the pI and MW values in the Spot Report or Cursor Information window should be the same as in the defined pI_MW annotations. However, this is only the case if the annotations are attached to actual spots and not just to pixel positions in the image. If an annotation is attached to a pixel, the pI and MW values for the spot that lies closest to it will be slightly different from that of the pixel (to which the annotation is attached). You can solve this ambiguity by linking the annotation to the spot.

6.8 Spot reports

6.8.1 Spot Table

The Spot Table (Figure 6-8), obtained by choosing ***Reports > Spot Table***, displays summarized information about enabled spots:

- Name of the image on which they were detected.
- Spot ID (see below), and Match ID if the spot was matched.
- Coordinates of the spot's center of gravity (X and Y).
- Quantification values: Intensity, Area, Vol, and %Vol. Depending on the spot detection algorithm used, the Saliency, Vol Ratio, and Slope will also be given.
- Calculated pI and MW values, if pI_MW annotations were defined on the image, or a matched image.
- All linked labels and spot sets.



	FileName	SpotID	Match ID	Pixel X	Pixel Y	pI	MW	Intensity	Area	Vol	%Vol	Saliency	Anova p<0.001
2198	A_T1_Gel3	136	392	857	275	-	-	1590	266	163684	0.0309635	408.192	☐
2199	A_T1_Gel3	135	693	531	274	-	-	5466	258	526442	0.099585	2552.47	☑
2200	A_T1_Gel3	134	1290	483	270	-	-	1220	259	143642	0.0271722	409.032	☑
2201	A_T1_Gel3	133	393	499	270	-	-	1922	383	287018	0.0542941	727.33	☐
2202	A_T1_Gel3	132	391	561	267	-	-	4558	273	473676	0.0896035	2020.34	☑

Figure 6-8. Spot Table.

Spot ID

Each spot in a gel has a unique identifier, called the Spot ID. Spot IDs of deleted spots are not reused. Melanie attributes a new ID to each new spot. When a spot is split, the child spot for which the coordinates are closest to the parent spot keeps the existing spot ID, the other child spot gets a new ID. When two spots are merged, the resulting spot is attributed the ID of the initial spot that was closest to the new center of gravity.

6.8.2 Cursor information

The Cursor Information window is available from the menu ***View > Global > Cursor Information Window*** or by clicking the corresponding icon in the Display toolbar. It can be used at any time to display information on pixels and spots located at the position of your mouse cursor (Figure 6-9).

Information on the pixel under the cursor:

- Name of the image.
- Calibrated pixel intensity.
- X and Y coordinates, expressed in pixels, in pI and MW units (if available), and in cm or inches.

Information on the spot under the cursor:

- Spot ID.
- Quantification values: Intensity, Area, Vol, and %Vol. Depending on the spot detection algorithm used, the Saliency, Vol Ratio, and Slope are also provided.

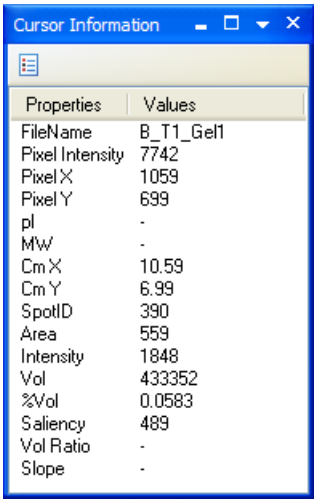


Figure 6-9. Cursor Information window.

7 Matches

7.1 Introduction

You know how to create an efficient match hierarchy, possibly with different levels of match sets, and to display this hierarchy in a sheet. You can also detect spots in all the gels in such a hierarchy. You are now ready to initiate the matching process itself.

Matching is a key operation in 2-DE image analysis. Basically, the image matching algorithm compares gel images to find matches between related spots, that is, spots representing the same protein in the gels. A match is therefore composed of spot n-tuples (S1, S2, ..., Sn) where S1 is a Spot ID in the first gel, ..., and Sn a Spot ID in the last gel.

The matching algorithm always starts from the reference image or match set, and looks for each spot in this reference, if corresponding spots in the other images are found. If a spot is absent from the reference, it cannot be matched automatically. However, if you have several match sets in your hierarchy, there is a good chance that the spot is on the reference of at least one of them. If so, the spot will turn up in the analysis. Subsequently, additional spots can be matched to it manually (Figure 7-1).

Matches are propagated at each level of the hierarchy. This means that once all match sets are effectively matched, spots from one gel can be directly compared with those in any of the other gels.

CAUTION! A DIGE gel is an inherent match set for which the co-run images are automatically matched. To subsequently match different DIGE gels, proceed like any other match sets.

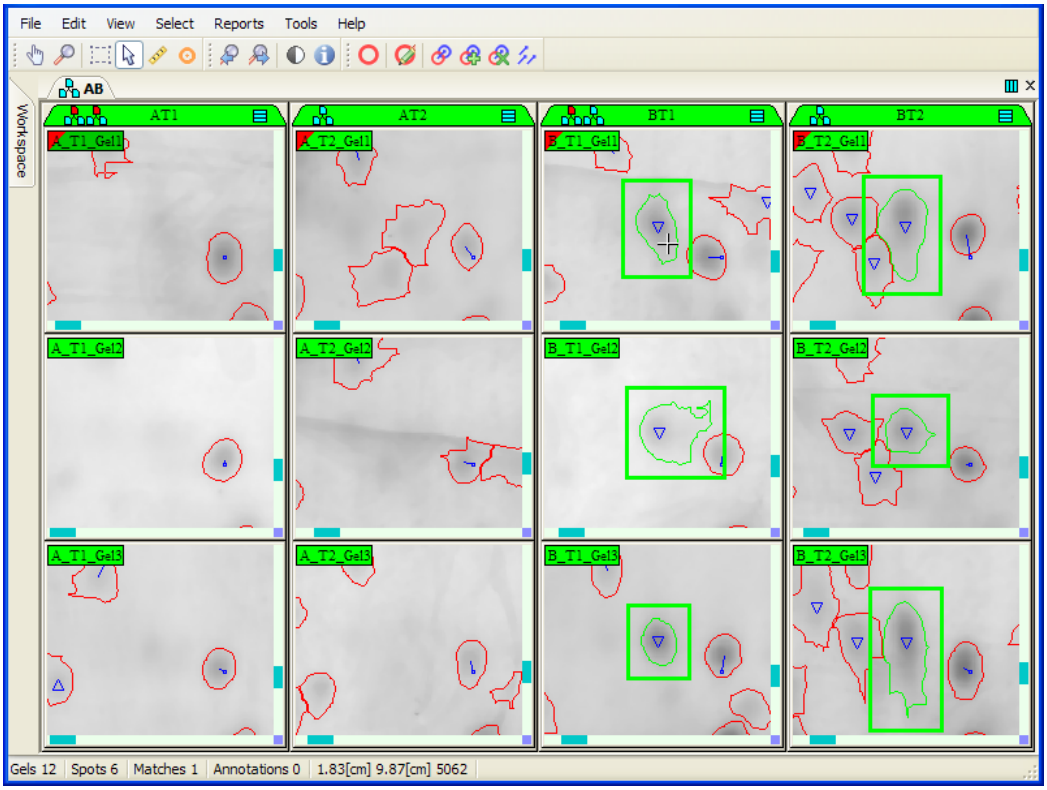


Figure 7-1. Reference and matching. The selected spot is absent from groups AT1 and AT2, including the sheet reference A_T1_Gel1. Since the spot is present in the references B_T1_Gel1 and B_T2_Gel1, it is matched to corresponding spots and turns up in the analysis (Gel and Class Analysis Tables).

7.2 Display a match hierarchy

In the example below, the match hierarchy AB was displayed by right-clicking on its name in the Workspace and selecting ***Display***. In the resulting sheet, only the lowest level (AT1, AT2, BT1, BT2) and highest level (AB) items are specifically visualized, in the panes and sheet, respectively. To select intermediary levels (A, B), you can use the corresponding icon in the pane tab of the reference (e.g., AT1) for that level (A).

At the same time, the red markers in these icons indicate what item in a given match level is used as the reference in the matching.

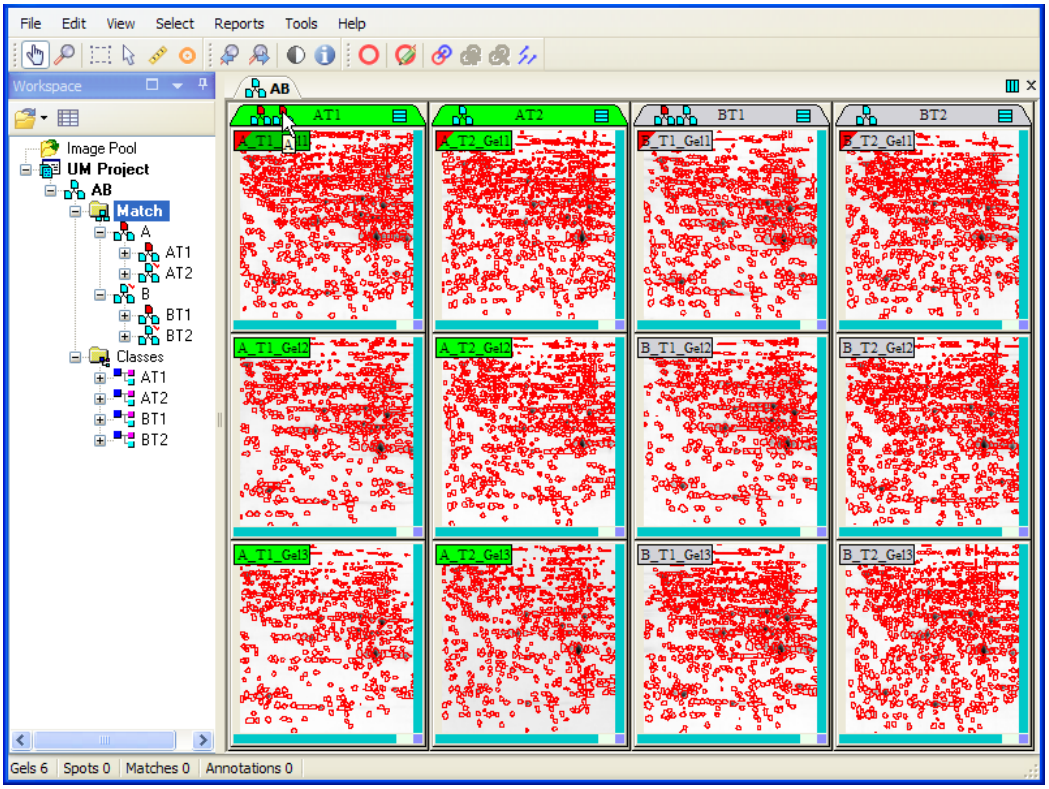


Figure 7-2. Match hierarchy AB displayed in a sheet. Note that the layout of the sheet and panes was changed to optimize the use of space and to get a better overview of the hierarchy. The match level A was selected by clicking on the corresponding icon in the pane tab of match set AT1.

7.3 Define landmarks

Melanie is designed to match gels with minimum user intervention. Nevertheless, when the gels are very distorted or different, you may need to help the matching process by specifying a few landmarks. Landmarks are points that relate corresponding spots in each of the gels to be matched.

We recommend trying to match your gels with only a single landmark or possibly none at all. In some cases, no landmarks are required. More often, a single landmark is sufficient for quick and efficient matching. If the matching results are not satisfactory, you can repeat the automatic matching procedure using additional landmarks.

The following rules should be considered when defining landmarks:

- Keep the number of landmarks to a minimum.
- Only define landmarks on clearly corresponding spots. Protein variants should definitely not be used as landmarks.
- Landmarks should be placed on small, sharp spots rather than on large, diffuse ones (to reduce the error in the position).
- Landmarks essentially correct global deformations of gels. Therefore, it is recommended not to put landmarks on spots in locally distorted regions because this can worsen the matching results. Rather place landmarks around such regions.

Landmark

Landmarks can be defined using the dedicated tool as described below.

To define landmarks:

1 Click the ***Landmark*** icon in the toolbar.

2 Place the mouse cursor over a known, well-defined spot in the first reference gel and click. A validated landmark symbol (bold orange circle) appears on the spot (Figure 7-3).

3 In the other images, drag the non-validated symbols (green circle with orange plus sign) onto the corresponding spots. If the symbol is already on a good spot, double-click to validate it.

4 Repeat steps 2 and 3 to add more landmarks.

In certain gels, the symbols only become visible once the landmark has been validated in the reference. In the example, the landmark must be validated in the image B_T2_Gel1 before any symbols appear in the images B_T2_Gel2 and B_T2_Gel3.

Sometimes, you may want to move or zoom your images during the landmarking process. When you click the Move or Zoom tools, the orange landmark symbols disappear, and only labels with the landmark numbers are left. When you click the ***Landmark*** tool again, the symbols are reactivated and you can continue defining landmarks.

To delete landmarks, you must delete the corresponding annotations. Choose ***Select > Annotations > By Category*** and select ***Landmarks*** in the list. Then choose ***Edit > Annotations > Labels > Delete***.

Figure 7-3. Placing of landmarks. Note the difference between the validated and non-validated landmark symbols.

In general, it is good to validate one or two landmarks on all the images in the hierarchy, so that they are used for matching at all levels. If it turns out after the matching that the gels within the lowest hierarchies (e.g., AT1, AT2, BT1 and BT2) have been properly matched, but that the higher level matches (e.g., A and B) are not satisfactory, you can add additional landmarks to the higher levels only. Do this by validating the landmark only on the reference images.

7.4 Automatic matching

An entire hierarchy can be matched automatically.

To match the gels in a match hierarchy:

1 Select the gels to be matched in the sheet. In principle, all the gels, even in a multilevel hierarchy, can be selected.

2 Choose ***Edit > Matches > Match Gels***. Or click ***Match Gels*** in the toolbar.

3 If several match sets are selected, you are asked to choose the ones to be matched in the Match Gels window (Figure 7-4). Items preceded by a ~ sign still require matching. Use the Ctrl or Shift keys to make multiple selections. All match sets can be selected at the same time. Click ***OK***.

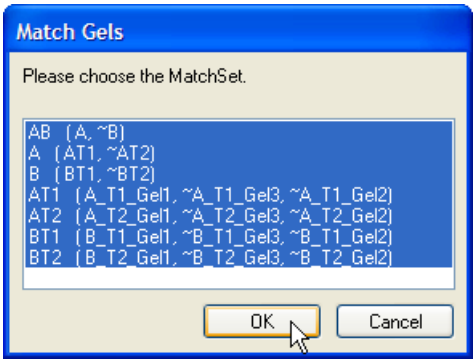


Figure 7-4. Match Gels window. In this example, all seven match sets were selected for matching. The items preceded by a ~ sign are not yet matched.

Match vectors are displayed in blue. The vector pattern is proof of consistency. If there is a mismatch, the vector has a different length and/or orientation.

If the matching did not work properly, you can rematch a particular match level after having added additional landmarks to the appropriate images.

7.5 Select matches

7.5.1 Select

When you select a spot with the ***Select*** tool, the matched spots are automatically selected.

Alternatives
There are other ways to select matches:

- Choose an option in the ***Select > Matches*** menu (All, Inverse Selection, Multiple Matches).
- Select matches in reports.

7.5.2 Match count

The software allows you to select spots present:

- In a certain number of gels: the ***Match Count*** in a ***Gel Analysis Table*** gives the number of gels in which the spot is present (detected and matched).
- In a certain number of classes: The ***Match Count*** in a ***Class Analysis Table*** gives the number of classes in which the spot is present (detected and matched).

Click the ***Select By Value*** icon in these tables to refine your selection. For example, select only spots that are present in at least X out of Y gels, or X out of Y classes.

7.6 Display matches

You can visualize matches by selecting them. The software also provides the following tools to display matches.

7.6.1 Show vectors

Right after matching, the software automatically displays the match vectors in blue. Vectors link the spots in a gel with the corresponding spots in the sheet reference. This sheet reference has a darker green gel name and should not be mistaken for the match reference, which has a red component. The sheet reference can be changed by choosing ***View > Sheet > Set Reference***.

A blue upside down triangle on a spot indicates that the spot was matched to one or more spots in other gels, but not to a spot in the sheet reference (Figure 7-5).

A spot with a triangle means that the corresponding position in the sheet reference lies outside the visible area (Figure 7-5).

To hide the match vectors, or on the contrary, to display them when they are not visible, choose ***View > Matches > Show Vectors***.

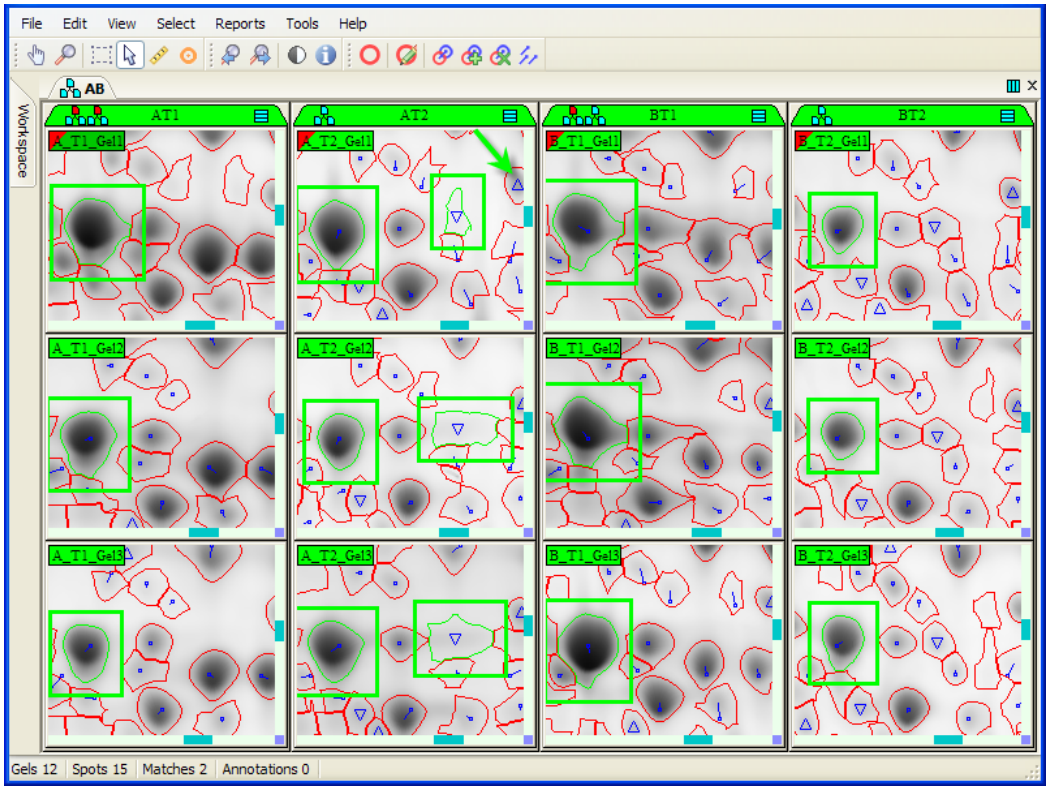


Figure 7-5. Different representations of matches. The selected spots in match set AT2 with a blue upside down triangle are matched, but not with a spot in the sheet reference. Spots with blue triangles, as the one indicated with a green arrow, are matched, but the corresponding position in the sheet reference lies outside the visible area.

To minimize the match vectors (after having moved or zoomed an image), select the ***Move*** tool and double-click in the image so that it is synchronized with the other images and the sheet reference.

You can change the default color for the match vectors by choosing ***Tools > Options*** and going to the ***Display*** tab.

7.6.2 Show ID

Choose ***View > Matches > Show ID*** to display Match IDs for selected spots on selected gels. Obviously, the gels must be matched. To hide the Match IDs again, possibly in selected gels only, choose ***View > Matches > Hide All ID***.

7.7 Edit matches

Sometimes it may be necessary to manually add or delete matches after the automatic matching procedure. Use the options in the ***Edit > Matches*** menu, or the corresponding icons in the Detect and Match Spots toolbar.

Note that matches were created in a hierarchical manner. This means that spots may be properly matched in one match set level, but not in another. To delete your matches, it is therefore important to select the appropriate images/panes.

Delete match

Select spots that were incorrectly matched, make sure the appropriate match level (gels/panes) is selected, and click the ***Delete Match*** icon to remove the spots from the match.

Add match

Select all spots to be matched and click the ***Add Match*** icon to add the spots to the match.

While selecting spots, you may see that some spots were already matched. When selecting one of them, the others are automatically selected. This makes it very easy to add matches. Nevertheless, you must make sure that the existing matches are correct. If this is not the case, delete them before proceeding.

Multiple matches

Melanie enables the creation of multiple matches (Figure 7-6). In contrast to a single match, where only a single spot is selected per gel, a multiple match implies that one or more spots in one image can be matched to several spots in other images.

All the spots from such a multiple match on a given gel image are considered as a single spot in the subsequent data analysis. The calculated quantification values for this composite spot reflect the size, intensity, and abundance of the combined spots. Therefore, this is an excellent solution to avoid spot editing.

Figure 7-6. Multiple matches. The selected spot was correctly matched throughout match set A and within BT2. But due to spot splitting in B_T1_Gel1, it was not matched within match sets BT1 and B. Rather than merging the two spots in B_T1_Gel1, the spots can both be included in the match and treated as a single entity in the spot quantification.

Review matches

To review matching, choose ***Select > Matches > All*** and specify the hierarchical level at which you want to select the matches. Matched spots are highlighted in green. The matching of any red spots should be scrutinized. However, this can also be done during data analysis.

7.8 Match reports

7.8.1 Match statistics table

Choose ***Reports > Analyze Gels > Match Statistics Table*** to display the number and percent of matches found for each of the images. By default, the numbers are calculated based on ***All MatchSets*** in the

hierarchy. But you can select a particular match set by clicking the ***Choose MatchSet*** icon in the toolbar of the Match Statistics Table (Figure 7-7).

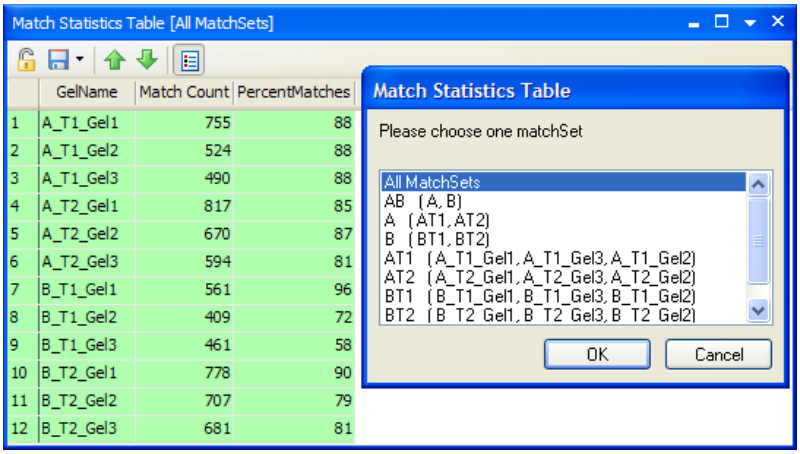


Figure 7-7. Match Statistics Table.

8 Data analysis

8.1 Introduction

To study the variations in protein expression among a series of gels, the gels should be matched together (be part of the same match hierarchy).

Data analysis can be carried out at two different levels:

- **Analyze Gels**: Study protein expression changes within a set of gels, without taking populations into consideration. This type of analysis can be carried out on both MatchSet and Classes sheets. The analytical methods used include scatter plots, descriptive statistics, histograms, and factor analysis.
- **Analyze Classes**: Find significant protein expression changes between classes of gels. For this type of analysis, images must be placed in classes and opened in a Classes sheet. The analytical methods used include descriptive statistics per class, histograms, overlapping measures, and statistical tests.

8.2 General settings

The following concepts and settings are used in the different analytical methods described later in this chapter.

8.2.1 Quantification value

The quantification value found in various tables, graphs and plots is a software option. Choose ***Tools > Options*** and go to the ***Quantification*** tab to set:

- **Value**: To be used for the analysis of conventional 2-D gels. You can choose between Intensity, Area, Vol, or %Vol. The default value is %Vol.
- **DIGE Value:** To be used for the analysis of DIGE gels. You can choose between Intensity, Area, Vol, %Vol, or Vol Ratio. The default value is Vol Ratio.

Note: *Although Vol Ratio are displayed in the tables, graphs and plots, the internal calculation of the statistical values (such as central tendency, dispersion and Anova) is based on the Log Vol Ratio.*

8.2.2 Statistics

Central tendency and dispersion are the most frequently-used descriptive statistics. They are calculated in Gel or Class Analysis Tables and Histograms.

These statistics are in fact numbers that summarize spot values from a match. The central tendency allows you to localize a center for the data, whereas the dispersion indicates how closely the data points fall around the center.

CAUTION! Absent spots, with zero values, are also considered in the calculation of statistics (for both central tendencies and dispersion).

Specify and display the statistics by clicking the ***Statistics*** icon in a report toolbar (Figure 8-1):

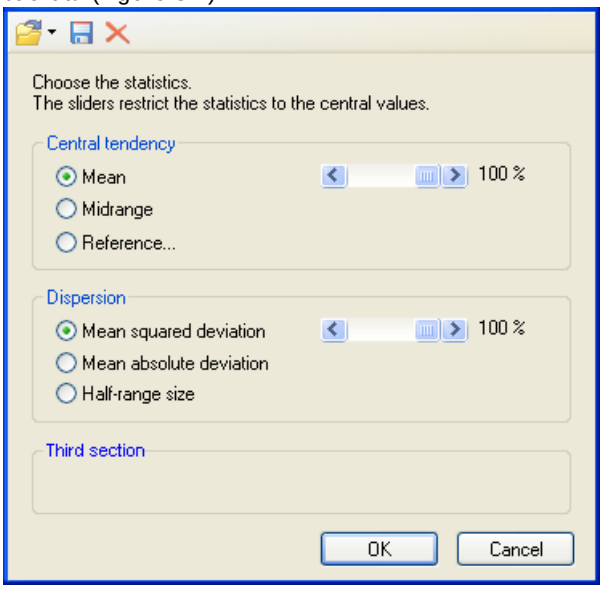


Figure 8-1. Statistics common to Gel or Class Analysis Tables and Histograms. The third section is specific to different report types and is described in the corresponding sections of the User Manual.

Central tendency

The central tendency gives the general location of a variable. This is commonly calculated by the arithmetic mean (also known as the average or center of gravity of a distribution), the median (the middle value which divides the sample in two equal parts) or the midrange (middle location between the two sample extremes).

Mean and midrange values are very sensitive to extreme values (outliers) and can be seriously affected by a single observation. On the other hand, the median is highly resistant to outliers. A compromise is given by the trimmed mean (or trimmed midrange) where a predefined number of outliers are removed from the sample. The trimmed measures are more robust than the mean (or midrange) but are more sensitive than the median.

The percentage slider in the Statistics window allows you to remove outliers and obtain the different central tendencies. A 100% value

means that all the spot values available in a match are used to calculate the statistics (no outliers are suppressed). With a value of 80%, for example, 10% of the minimum values and 10% of the maximum values are discarded from the sample and the trimmed measure is calculated with the remaining values.

- Select ***Mean*** and the arithmetic mean is calculated, that is, the sum of all the sample values divided by the sample size.
- Select ***Midrange*** and the midpoint of the sample value is calculated, that is, the middle location between the two sample extremes.
- Obtain a **Trimmed Mean** (or **Trimmed Midrange**) by selecting ***Mean*** (or Midrange) and discarding the desired percentage of outliers with the percentage slider.
- Obtain the **Median** by selecting ***Mean*** and discarding 50% of the values at each of the extremities, that is, select 0% in the percentage slider.
- Select ***Reference...*** and the value from the spot in a specified reference gel is taken as the central tendency. This option is only available for Gel Analysis Reports.

Dispersion

The dispersion measures the variability of the sample data as indicated by how clustered or scattered the data points are around their center value. There are numerous measures of variability: standard deviation, range, interquartile range, and so on.

Like the statistics for central tendency, these measures make use of all the available sample data and can be heavily influenced by outliers. Therefore, you can also restrict the sample to the central values by trimming out the extreme values with the percentage slider.

- Select ***Mean Squared Deviation (M.S.D.)*** and the square root of the average squared difference of each sample value to the center location is calculated.
- Select ***Mean Absolute Deviation (M.A.D.)*** and the mean of the absolute difference between each value and the central value is calculated. It is not affected as much by outliers as the Mean Squared Deviation because the differences are not squared.
- Select ***Half-range Size*** and the difference between the largest and the smallest values divided by 2 is calculated.

Examples

- The Mean 100% and the Mean Squared Deviation 100% are the most commonly-used statistics (Figure 8-2, a). Note that the standard deviation is the Mean Squared Deviation multiplied by $\sqrt{N/(N-1)}$, where N is the sample size. This difference comes from the fact that the standard deviation should be an unbiased estimator.

- The Median (Mean 0%) and Mean Absolute Deviation 100% are much more robust to outliers than the statistics above (Figure 8-2, b).
- The Midrange 100% and Half-range 100% define an interval that includes all sample values (Figure 8-2, c).
- The Midrange 50% and Half-range 50% are known as order statistics and interquartile ranges (Figure 8-3).

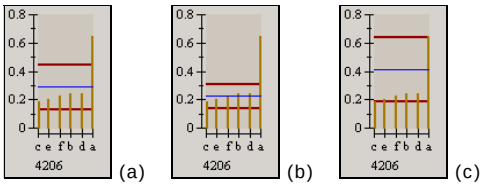


Figure 8-2. Histograms showing the sensitivities of central and dispersion values. (a) Mean 100% and M.S.D. 100%, (b) Median and M.A.D. (c) Midrange 100% and Half-range 100%.

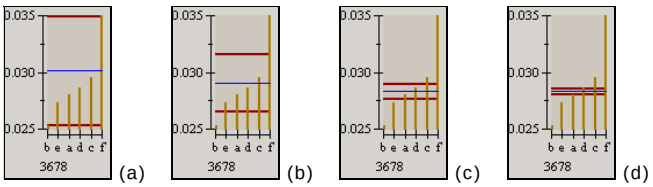


Figure 8-3. Histograms showing the effect of suppressing outliers. Midrange and half-range values are given for (a) 100%, (b) 80%, (c) 50% and (d) 33%.

8.3 Analyze gels

8.3.1 Scatter plots

To analyze gel similarities or experimental variations such as disparities in stain intensity or sample loading, you can produce Scatter Plots for matched spots (Figure 8-4) by choosing ***Reports > Analyse Gels > Scatter Plots***.

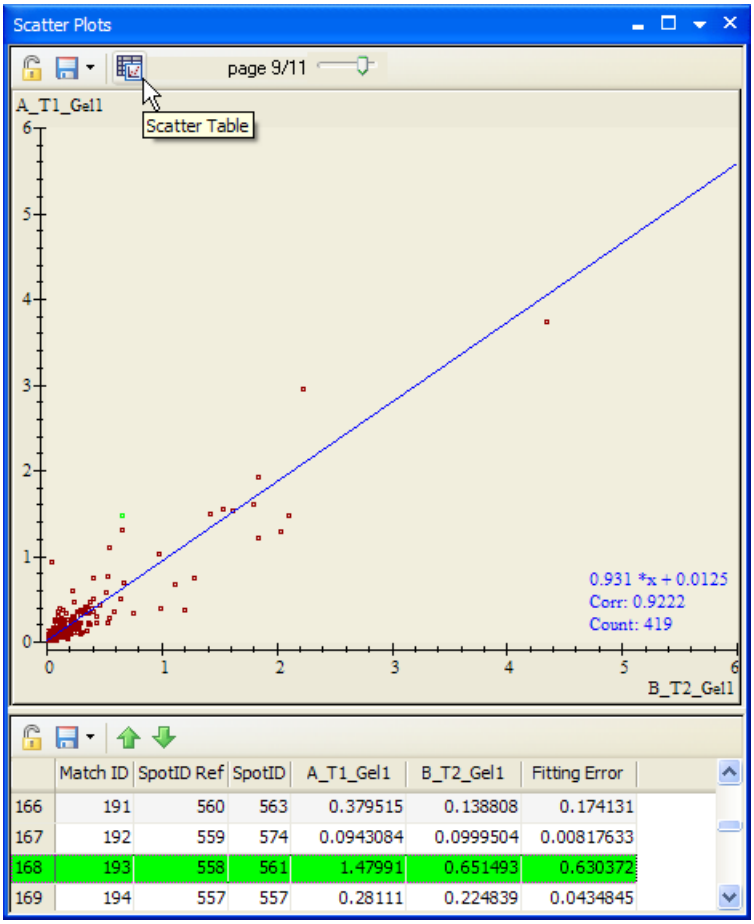


Figure 8-4. Scatter plot for matched spots.

Scatter plots give an idea of the relationship between the spot values from two gels by searching for the linear dependence between the spot values of one gel (variable X) and the corresponding values in the sheet reference (variable Y). Remember that you can change the sheet reference (darker gel name) by choosing ***View > Sheet > Set Reference***.

The linear dependence is defined as the best-fit line through the data points. The best-fit line is described by a slope and its offset from the equation y = slope × x + offset.

The goodness-of-fit for this approximation is given by the correlation coefficient ***Corr***. This coefficient can vary between -1 and 1, where an absolute value near 1 indicates a good fit. The spot values of one gel can be predicted, to some extent, by the values of the other gel. On the

other hand, a low value indicates that the data could not be approximated by a straight line.

The types of conclusions that can be drawn from the regression line equations and the correlation coefficients are:

1.0 × x + 0	and Corr = 1	indicates that the spot values for all matched spots are the same in the two gels.
1.2 × x + 0	and Corr = 0.95	indicates that almost all spot values are approximately 20% higher in the sheet reference.
1.0 × x + 0.2	and Corr = 0.95	indicates that almost all spot values are shifted by +0.2 with respect to the sheet reference.

In general, when the data are highly correlated (Corr close to 1) but the best-fit line is far from identity (1.0 × x + 0), you should search for possible reasons to explain why your values are systematically biased. Stain intensity variations, differences in protein loading, or image acquisition problems are some typical causes.

In the Scatter Plots window you can visualize a scatter plot for each gel in the sheet versus the sheet reference, together with the best-fit line, correlation coefficient and the number of matches displayed.

Scatter plots are interactive. You can click on the points representing the matched spots. This in turn selects the spots in the gels and other reports.

Slider

Move the slider in the toolbar of the Scatter Plots window to view the scatter plots for the other images in the sheet.

Scatter table

Click the ***Scatter Table*** icon in the toolbar to show or hide the table below the plot. The Scatter Table displays, for each pair of matched spots in the scatter plot, the corresponding spot values in the gels and the error in relation to the regression line.

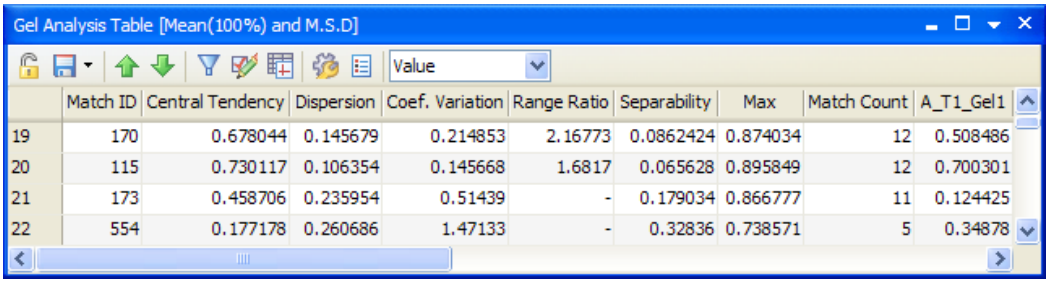
Copy formula to clipboard

Select the ***Copy Formula to Clipboard*** option from the ***Save*** icon in the drop-down list to copy the regression formula and correlation coefficient to the clipboard for use in other applications.

8.3.2 Gel analysis table

The Gel Analysis Table and Histograms provide valuable tools for looking at unusual matches within a set of gel images. Analyzing protein expression changes, checking spot detection or verifying matching operations are just a few of the potential uses. Choose ***Reports > Analyse Gels > Table*** to display the Gel Analysis Table (Figure 8-5). This report describes, for each match:

- The spot values from each gel.
- The ***Max*** value, that is, the highest of all these spot values.
- The ***Central Tendency*** and ***Dispersion*** over all the gels in the sheet, regardless of whether they belong to different populations or not.
- The ***Coefficient of Variation*** (***Coef. Variation***), which is the dispersion divided by the central tendency. It measures the relative variability of the spots in a match by correcting for the magnitude of the data values, thus giving a measure that has no units. When you choose the Median and Mean Absolute Deviation statistics, this measure is also known as the Coefficient of Dispersion.
- The ***Range Ratio***, which is the maximum value divided by the smallest value in the sample specified. To specify the sample, click the ***Statistics*** icon in the Gel Analysis Table toolbar, and suppress outliers by setting the percentage slider in the ***Range Ratio*** section to the desired value.
- The ***Separability***, which is the highest difference between two consecutively sorted values in the whole sample. It measures the greatest gap that you can have if you want to split the spot values in a match into two separate classes.
- The ***Match Count***, which is the number of gels in which the spot is present and matched.



	Match ID	Central Tendency	Dispersion	Coef. Variation	Range Ratio	Separability	Max	Match Count	A_T1_Gel1
19	170	0.678044	0.145679	0.214853	2.16773	0.0862424	0.874034	12	0.508486
20	115	0.730117	0.106354	0.145668	1.6817	0.065628	0.895849	12	0.700301
21	173	0.458706	0.235954	0.51439	-	0.179034	0.866777	11	0.124425
22	554	0.177178	0.260686	1.47133	-	0.32836	0.738571	5	0.34878

Figure 8-5. Gel Analysis Table.

In addition to the standard functionalities for saving, printing, copying content to the clipboard and navigating in the report, the following tools in the Gel Analysis Report are particularly useful:

Select by value

Select items in the table based on a numerical search criterion. Choose the measure (i.e., column) to be used for refinement, and set the lower and/or upper limits of your search interval.

Create spot set

Create a spot set to annotate spots of interest for use at a later stage. All currently selected spots are automatically included in the newly created spot set, which appears as a column in the table. You see a checked box for spots that belong to the set, or an empty box for spots that do not belong to the set.

Factor analysis table

Carry out a factor analysis.

Statistics

Set the statistics to be used in the report. These settings are common to the Gel Analysis Table and Gel Analysis Histograms.

Settings

Some of the above-mentioned columns may not be displayed by default. Click the ***Settings*** icon to show or hide columns in the table.

Normalization

In order to simplify comparisons across matches, the spot values in the Gel Analysis Table can be normalized relative to their gel analysis statistics. Select the desired type of normalization in the drop-down list in the toolbar. The following options are available:

Value	Raw spot value.
Relative	Spot value – Central tendency
Ratio	$\frac{\text{Spot value}}{\text{Central tendency}}$
Normalized	$\frac{\text{Spot value} - \text{Central tendency}}{\text{Dispersion}}$

CAUTION! *The Relative and Normalized options are not available for DIGE experiments when Vol Ratio is selected as the quantification measure.*

8.3.3 Gel analysis histograms

Histograms are a way to look at matched spots. The Gel Analysis Histograms window (Figure 8-6) is displayed by choosing ***Reports > Analyze Gels > Histograms***.

In the histograms, the vertical orange bars correspond to the spot values, the blue horizontal line represents the chosen central tendency and the red lines delimit the range defined by [Central value - Dispersion, Central value + Dispersion]. The Match ID is displayed at the bottom of each histogram.

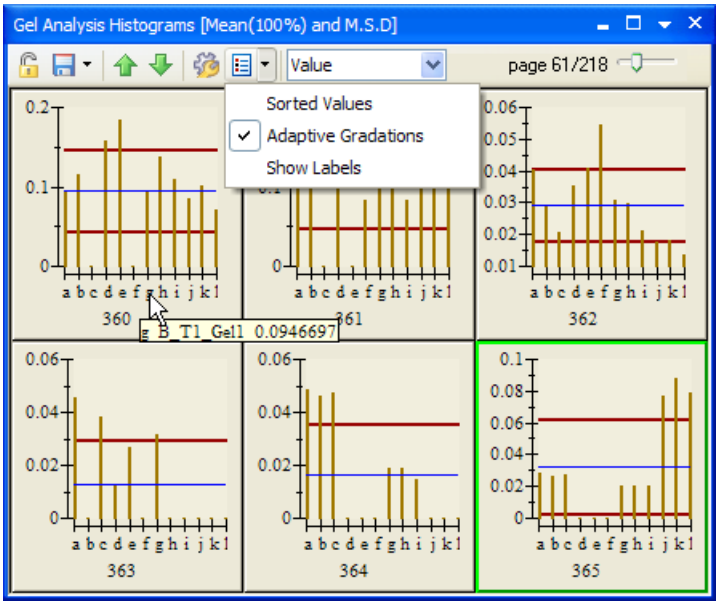


Figure 8-6. Gel Analysis Histograms.

The following tools in the Gel Analysis Histograms are very useful:

Slider
To see the matches displayed on additional pages, use the slider in the toolbar. When a match is selected on an image or in another report, it is automatically highlighted in a histograms window.

Statistics
Set the statistics to be used in the histograms. These settings are common to the Gel Analysis Table and Gel Analysis Histograms.

Settings
Click the ***Settings*** icon to select one of the following options:

- ***Sorted Values***, to sort the spot values in ascending order (Figure 8-7).

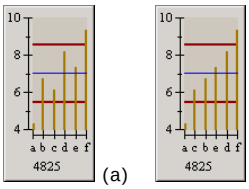


Figure 8-7. Histograms on matches with (a) unsorted values and (b) values sorted in ascending order.

- ***Adaptive Gradations***, to adjust the histogram gradations according to the spot values in each match. Deselect this option to display an identical gradation in all histograms (Figure 8-8).

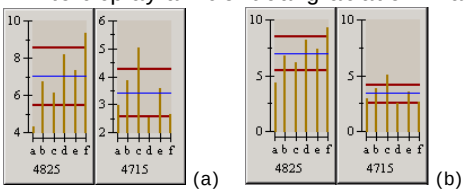


Figure 8-8. Histograms on matches with adaptive gradations set (a) individually for each histogram and (b) according to the minimum and maximum values in all histograms.

- ***Show Labels***, to display a table with the gel names. Alternatively, when you place the mouse over a letter in the histograms, a screentip displays the full image name and the spot value in the image.

Normalization

You can display normalized spot values to simplify the comparisons across matches. These normalized values are particularly useful in combination with the histograms:

Value	Raw spot value.
Relative	Spot value – Central tendency This normalization sets the central tendency values to 0, and if the Adaptive Gradations option (see above) is deactivated, you have a good overview of the dispersion and therefore of the homogeneity of the matches. This normalization is sensitive to high spot values.

Ratio	$\frac{\text{Spot value}}{\text{Central tendency}}$ This normalization divides all values by the central tendency and thus gives a ratio for all data. If you deactivate the Adaptive Gradations option (see above), all histograms have the same scale and thus it becomes easier to detect matches that do not have homogenous values. This normalization is more sensitive to low spot values.
Normalized	$\frac{\text{Spot value} - \text{Central tendency}}{\text{Dispersion}}$ This normalization is a compromise between the two normalizations described above and is sensitive to all values.

CAUTION! *The Relative and Normalized options are not available for DIGE experiments when Vol Ratio is selected as the quantification measure.*

8.3.4 Factor analysis

The visual task of comparing gels is rather difficult when dealing with a large number of gels that consist of thousands of spots. It can be hard to assess whether different sample populations exist and to characterize their different protein expression profiles. Factor analysis helps here by condensing the information contained in such huge data sets into a smaller number of factors, or dimensions, that explain most of the variance observed. The factor analysis tool is used to examine the interrelationships between large numbers of variables (i.e.,, spot values for a series of gels) and to explain these relationships (e.g., gel populations) in terms of common underlying factors (or associations with specific spot patterns).

Factor analysis is a complex statistical technique, whose comprehensive description is beyond the scope of this manual. For more information, references are given in the Appendix.

Perform a factor analysis

A factor analysis is carried out on all or selected matches in the Gel Analysis Table. You must judge which of the options described below is most applicable to your analysis.

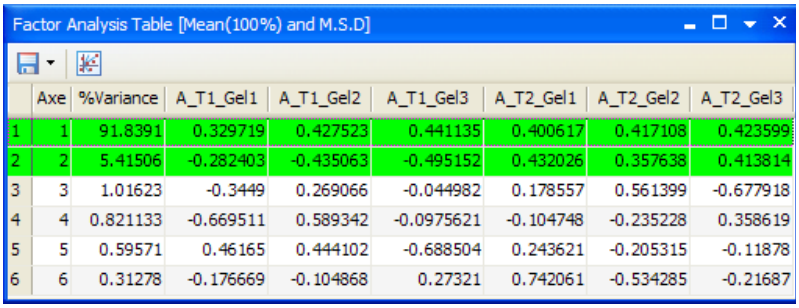
To carry out a factor analysis:

1 Click the ***Factor Analysis Table*** icon in the Gel Analysis Table toolbar.

2 If any matches are selected, the software asks whether you want to use all or only the selected rows.

3 The Factor Analysis Table (Figure 8-9) is displayed with the lines corresponding to the axes that can be drawn in the Factor Projection Plot. Select two axes to be displayed in this plot (generally the first two ones).

4 Click the ***Factor Projection Report*** icon in the Factor Analysis Table toolbar.


Factor Analysis Table [Mean(100%) and M.S.D]

	Axe	%Variance	A_T1_Gel1	A_T1_Gel2	A_T1_Gel3	A_T2_Gel1	A_T2_Gel2	A_T2_Gel3
1	1	91.8391	0.329719	0.427523	0.441135	0.400617	0.417108	0.423599
2	2	5.41506	-0.282403	-0.435063	-0.495152	0.432026	0.357638	0.413814
3	3	1.01623	-0.3449	0.269066	-0.044982	0.178557	0.561399	-0.677918
4	4	0.821133	-0.669511	0.589342	-0.0975621	-0.104748	-0.235228	0.358619
5	5	0.59571	0.46165	0.444102	-0.688504	0.243621	-0.205315	-0.11878
6	6	0.31278	-0.176669	-0.104868	0.27321	0.742061	-0.534285	-0.21687

Figure 8-9. Factor Analysis Table.

The Factor Projection Plot (Figure 8-10) displays the projection of each match (cross) and each gel (blue vector) on the two factorial axes. The blue curve represents a part of the correlation circle; its form is linked to the scale of the axes. When matches are selected on the plot, they are automatically highlighted on the gels and/or any open reports.

The Factor Projection Table (Figure 8-10) displays the contribution of each match to the two axes displayed in the Factor Projection Plot. The ***Quality*** measures whether the projection of the match is well represented on the factorial subspace.

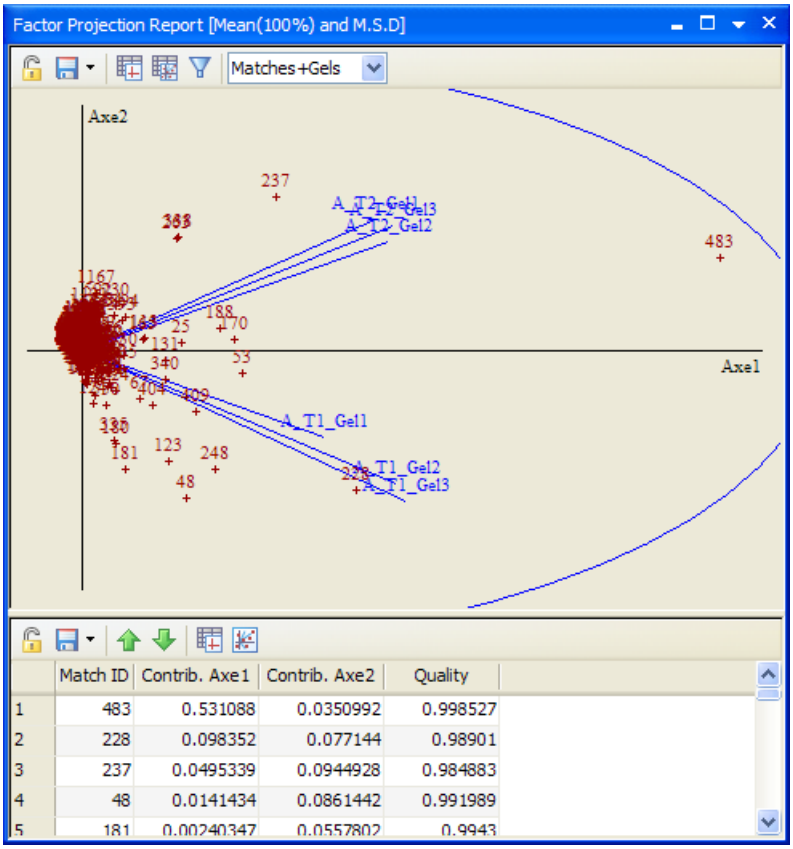


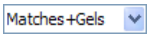
Figure 8-10. Factor Projection Report includes the Factor Projection Plot (top) and the Factor Projection Table (bottom).

You can use the following tools in the Factor Projection Report:

Factor analysis table
Display the Factor Analysis Table from which the two axes for the current Factor Projection Report were selected.

Factor projection table
Show or hide the Factor Projection Table corresponding to the displayed plot.

Factor projection plot
Show or hide the Factor Projection Plot corresponding to the displayed table.



Displayed items

Choose the items to be displayed from the drop-down list in the toolbar of the plot. You can choose matches (crosses), gels (blue vectors), or both.

Matches displayed

Enter the number of matches to be displayed in the plot.

Interpret a factor analysis

How to interpret a factor analysis is explained with an example of six gels run to compare the effect of two treatments T1 (A_T1_Gel1, A_T1_Gel2, A_T1_Gel3) and T2 (A_T2_Gel1, A_T2_Gel2, A_T2_Gel3) on bacteria cultivated on substrate A. The gels were detected and matched, and a Gel Analysis Table displayed.

The Factor Analysis Table (Figure 8-9) summarizes the variance accounted for by successive axes (or factors), expressed as a percent of the total variance. Thus, factor 1 accounts for 91.8% of the variance, factor 2 for 5.4%, and so on. The coordinates for each gel projected on these axes is also listed.

The number of factors equals the number of gels being analyzed. Factor analysis cannot be performed with less than two gels and so at least two factors are always calculated. Of course, the more gels you use, the more reliable the results are likely to be, and the more factors are calculated. Since the first factors are generally the best ones for characterizing gels and matches that behave similarly than any subsequent ones, the factors are ranked in order of importance.

Figure 8-11 shows the Factor Projection Plot obtained when the first two axes in Figure 8-9 were selected. In the example, only the 20 most significant matches are displayed on the projection plot. If all matches were shown, one would find that many of them cluster around the origin of the graph. This illustrates that the majority of matches, i.e., protein spots, are not meaningful in classifying the gels. The further away a spot is from the origin, the more important it is likely to be in terms of characterizing the gels.

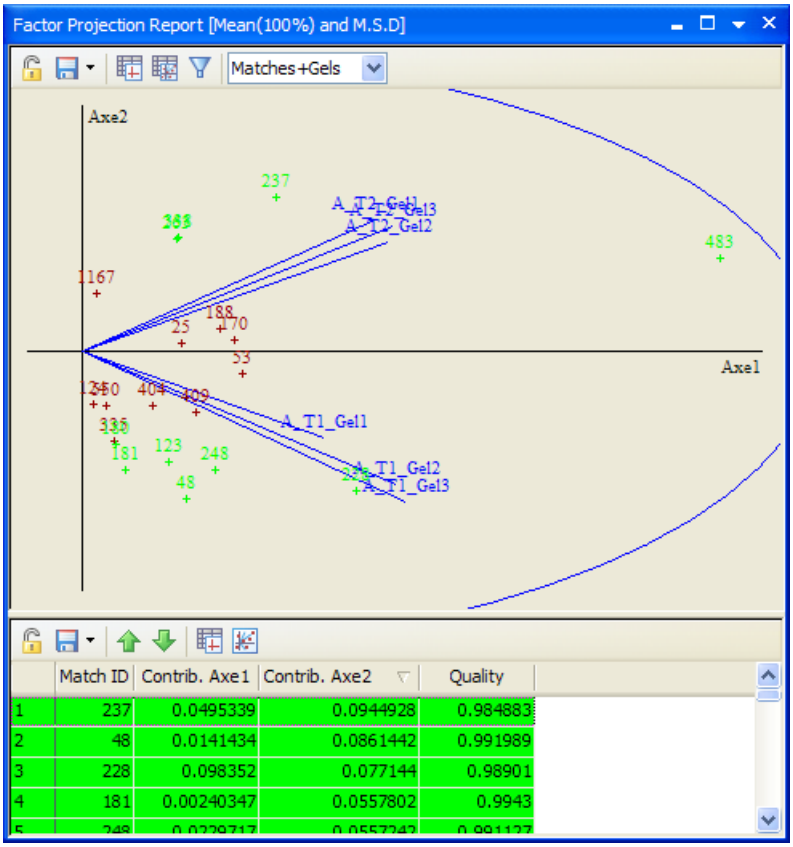


Figure 8-11. Factor Projection Report. The ten matches with highest contribution to the second axis are selected.

To find a possible meaning for a given factor, one should first identify the matches that largely contribute to this factor. The Factor Projection Table is used for this purpose. When sorting the matches according to their contribution to the first axis, one discovers that the matches at the top of the table are those with the highest relative volumes, as found in the Gel Analysis Table. In fact, the first axis is generally correlated with protein abundance.

Please note that the Factor Projection Table also contains the Quality for each match. This number tells you how close the distance of the projection is to reality. Matches with very similar behavior (similar expression profiles across gels) are close in space. However, when projected onto a two-dimensional subspace, matches that are actually far apart may appear close together. It is therefore important to look at the Quality to judge whether matches are effectively close. If the values are high for both matches, the chance is great that they are indeed

nearby and have a similar behavior. If one of the matches has a low value, any interpretation becomes tentative.

Gels that are adjacent on the graph are likely to be similar to each other. They may correspond to the same population. This is clearly the case in the example above. The gels from T1 cluster together below the horizontal axis, whereas the gels corresponding to T2 lie above.

The closer a match is to a given set of gels, the more characteristic it is likely to be of those gels. That is, the more important the match is in determining why those gels are different from other gels. In Figure 8-11, one can observe that the matches 48, 123, 180, 181 and 248 are close to the gels belonging to T1. The histograms in Figure 8-12 show that these matches have high spot values for the T1 gels and low values for the T2 gels. Matches 237 and 338, on the other hand, are characteristic of the T2 population, with higher spot values in the T2 gels. In our example, the second axis appears very important for separating the gels into two classes. It is related to the ratio between the mean spot values in each population of gels. The matches in the upper part of the graph have ratios that favor the T2 population, while those below the horizontal axis have ratios that favor the T1 gels.

Comments on factor analysis

Factor analysis is used to examine the protein expression pattern within each match. The quality of the output depends on the quality of spot matching. Hence, it may be useful to exclude spots that are not well matched across all gels (using ***Match Count*** in the Gel Analysis Table). Nevertheless, in cases where a majority of spots were properly matched, the inclusion of all matches in the factor analysis can yield good results with no preliminary match filtering necessary.

This statistical method, based on data variation and their standard deviations, highlights the natural formation of populations among the gels and allows identification of matches (i.e., matched spots) that are characteristic of these classes. However, one should be very critical when analyzing factor analysis plots since the results can be greatly influenced by outliers, bad matches, and so on. Factor analysis can provide valuable indications in some cases, but not in others.

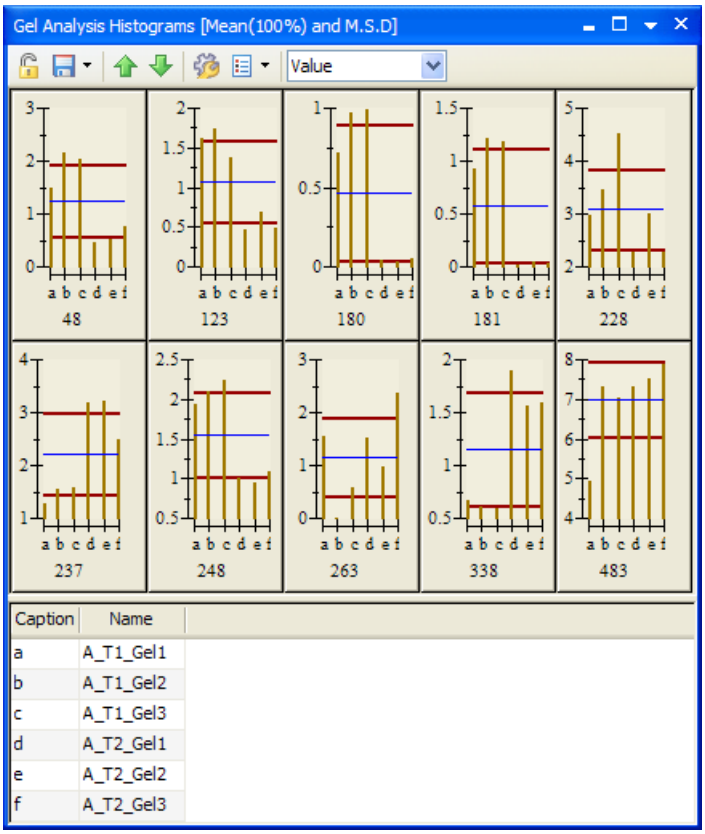


Figure 8-12. Gel Analysis Histograms for the ten matches ranked highest for their contribution to the second axis in the factor analysis example. Matches 48, 123, 180, 181 and 248 are characteristic of population T1, matches 237 and 338 are characteristic of population T2.

8.3.5 DIGE histogram

A DIGE Histogram can be displayed when detected DIGE gels are selected. It shows, for each non-reference image, data associated with detected spots in the image, plotted against Log Volume Ratio on the X-axis (Figure 8-13). It has two different Y-axes:

- The left Y-axis displays the spot frequency. The blue curve represents the frequency distribution of the log volume ratios.
- The right Y-axis represents the Measure parameter (see below) selected from the ***Measure*** tool in the DIGE Histogram window. A plotted single data point on the histogram represents an individual protein spot.

The name of the DIGE reference for the current image, used for the calculation of the Log Volume Ratio, appears at the lower right corner of the DIGE Histogram window.

The DIGE Histogram is dynamic. You can click on the data points representing spots to select them on the gels and any other open reports.

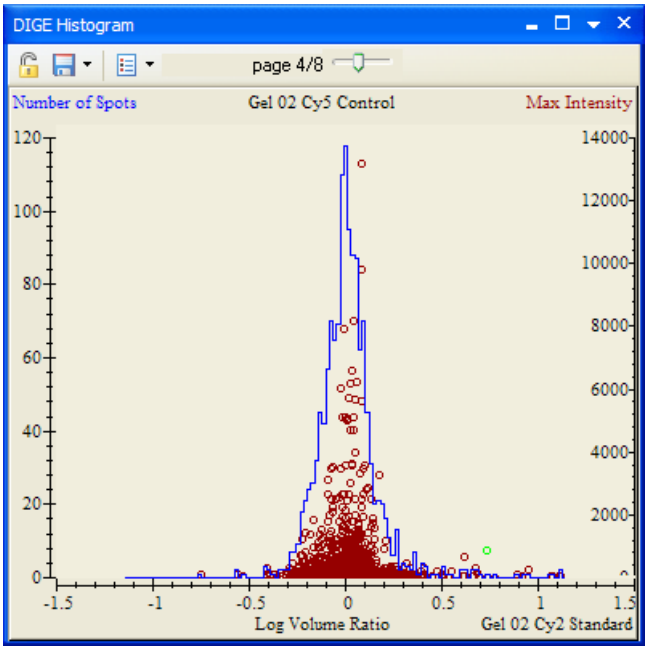


Figure 8-13. DIGE Histogram.

Two report-specific tools are available:

Slider

Move the slider in the toolbar of the DIGE Histogram window to view the histograms for the other images in the sheet.

Measure

Select one of the following options from the ***Measure*** icon:

- ***Max Slope***: Largest gradient associated with the co-detected spots.
- ***Area***: Number of pixels within the spot boundary.
- ***Max Intensity***: Largest pixel value associated with the co-detected spots.
- ***Max Volume***: Volume of the largest co-detected spot.

8.4 Analyze classes

8.4.1 Specify classes

To identify protein expression variations between populations of gels, you need to specify what gels belong to which population by creating classes. Classes are created in the Workspace.

Oftentimes, you already know the populations for your set of gels. This is the case when you are comparing gels from healthy tissue extracts with those from disease-associated samples. When you have no preliminary knowledge of the populations in the set of gels, you can draw possible conclusions from factor analysis.

8.4.2 Overlapping measures

Spot values for a given match within each class can be summarized by the central tendency and dispersion. In addition to these descriptors, the software computes overlapping measures between the class intervals, where class intervals are defined by the ranges [Central value - Dispersion, Central value + Dispersion].

The overlapping measures quantify the overlap between these intervals, and thus gauge how different the protein expression changes between the classes really are. They take into account both the difference between the central tendency in each population and the dispersion. The following overlapping measures are available:

- ***Gap***: Maximum difference between the range of the current class and the range of one of the other classes (Figure 8-14, c-b in the case of Class A). Negative values indicate overlap whereas positive values are non-overlapping class ranges.
- ***Ratio***: Maximum ratio between the lower limit of one of the other classes and the upper limit of the current class (Figure 8-14, c/b in the case of Class A). Absolute values smaller than 1 indicate overlap, whereas absolute values higher than 1 show that there is no overlap. In order to easily distinguish matched spots that are over or under expressed in one of the classes, the ratio value is preceded by a minus sign when the protein spot is under expressed for the class in question, compared to the other class. Positive values are attributed to the Ratio value in the over-expressed class.
- ***Normalized***: Maximum percentage of the current class range not overlapping with the range of one of the other classes (Figure 8-14, (c-a)/(b-a) in the case of Class A). A value smaller than 1 indicates overlap. For example, 0.25 implies that 25% of the current class range is not recovered by one of the other classes. In the same way, a value of 1.5 indicates that there is a gap equivalent to 50% of the current class range to the furthest other class. The normalized overlapping is not symmetrical, the value from Class A

compared to Class B is not the same as the value from Class B compared to Class A.

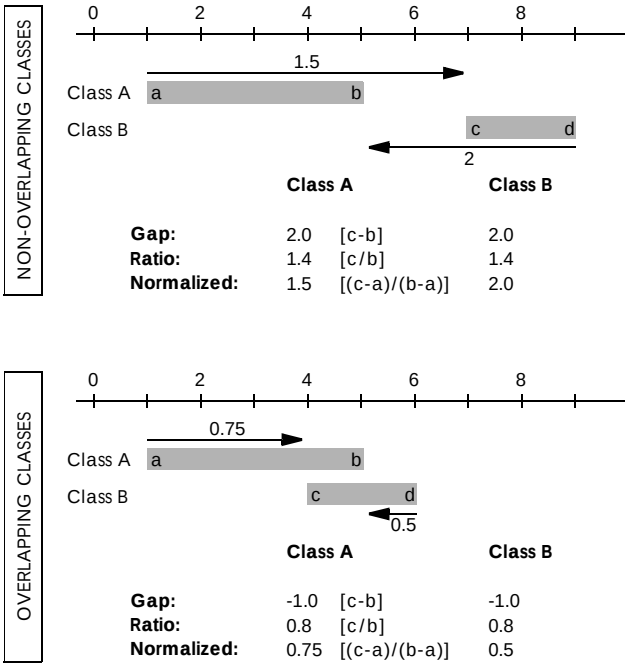


Figure 8-14. Scheme demonstrating how the Gap, Ratio, and Normalized values are calculated for two non-overlapping classes (upper part) and two overlapping classes (lower part). Arrows above or below each class range illustrate how the Normalized measure relates to this class range.

Please note that the formulas mentioned above only apply to Class A in the current example. Their purpose here is to illustrate the principles of overlapping measures. Many different cases (and therefore formulas) exist.

A value of 1e6 (1000000) in the Class Analysis Histograms characterizes the case where the protein is completely absent from a class. The software cannot compute ranges. A value of 0 for the Ratio or Normalized measures indicates that the particular class is entirely covered by another one.

CAUTION! *The Gap, Ratio and Normalized options are not available for DIGE experiments when Vol Ratio is selected as the quantification measure.*

CAUTION! As indicated in their definition, the Gap, Ratio, and Normalized values always calculate the MAXIMUM difference, ratio or percentage with respect to ANY of the other classes. This means that when the Ratio values for three populations (e.g., A, B, C) are compared, the software calculates the ratio of A with respect to B and of A with respect to C, but only displays the highest value in the column for class A. The number shown does not indicate with respect to which class the value was obtained. The idea is to quickly enable you to find a match (i.e., protein marker) that distinguishes the current class from any of the other classes. Once such protein markers are found, the Class Analysis Histograms can be used to study the match in more detail.

8.4.3 Fold change

Instead of using overlapping measures between the class intervals, you may simply want to look at the fold change between the central tendency of one class and the central tendency of one or several other classes, ignoring the dispersion. Melanie provides two options:

The ***Fold*** value displays the fold-change between the class with the lowest central value and the class with the highest central value. It is available in a separate column of the Class Analysis Table (see 8.4.5 Class analysis table).

It is possible to display, for each class, the fold-change against a selected reference class X. That is, the ratio of the central tendency for the class versus the central tendency for the selected reference class. To display these ratios, select the option ***versus Class X*** in the Displayed value drop down of the Class Analysis Table that corresponds to the desired reference class X (see 8.4.5 Class analysis table).

8.4.4 Statistical tests

The software provides three statistical tests: ANOVA, Mann-Whitney U test, and the Kolmogorov-Smirnov test. These tests are used to analyze differences in protein expression between classes of gels. The idea is to draw conclusions about the significance of the protein expression changes by extrapolating information from the data you collected. For example, when you have two samples (classes) with different means (i.e., different means for the spot values of a particular match), you might want to know whether the data were sampled from populations with different means or whether the populations have the same mean with the observed difference being a coincidence of random sampling.

In fact, there is no way to definitely conclude which of the two possibilities is true. All you can do is calculate the probability of observing a certain difference (or larger) between sample means in an

experiment of this size, for populations that in reality have the same mean. If the probability is small, you can conclude that the difference is not likely to be caused by random sampling and assume instead that the populations have different means.

CAUTION! You can display the desired statistical values for each match in the Class Analysis Table. These values should be considered as qualitative indications of the variations in protein expression between two populations. To draw quantitative conclusions, you must verify that the restrictive assumptions of the various tests are met. In addition, one should always check the results by visual inspection of the spots, since the conclusions may be erroneous due to inaccuracies in detection or matching.

CAUTION! The given statistical values are useless when the samples (classes) do not consist of more than two gels. Your objective should always be to work with the largest possible sample sizes.

One-way ANOVA

Analysis of Variance (ANOVA) is one of the most important statistical tests available for biologists. It is essentially an extension of the logic of Student's t-tests to those situations where the comparison of the means of several groups is required. Thus, when comparing two means, ANOVA gives the same results as the t-test for independent samples.

One-way ANOVA tests the null hypothesis that all populations have identical means. It generates a P value that answers this question: If the null hypothesis is true, what is the probability that randomly selected samples vary as much (or more) than actually occurred?

It is based on the same assumptions as the t-test:

- The samples are randomly selected from, or at least representative of, the larger populations.
- The two samples are independent. There is no relationship between the individuals in one sample as compared to the other.
- The data are sampled from populations that approximate a Gaussian distribution.

If you are not able to assume that your data are sampled from Gaussian populations, then non-parametric tests like the Mann-Whitney or Kolmogorov-Smirnov tests can provide a better analysis. Please note that these test only allow you to compare two samples at the same time.

Mann-Whitney or Wilcoxon test

The Mann-Whitney U test or rank sum test is the non-parametric substitute for the two-sample t-test when the assumption of normality (Gaussian bell-shaped distribution) is not valid. It is equivalent to the Wilcoxon rank sum test. It should only be used for comparing two unpaired samples. The assumptions of the Mann-Whitney U test are:

- The variable of interest is continuous (not discrete) and the measurement scale is at least ordinal. This means that repeated values (ties) are not acceptable. When ties are present in your data, there is an approximation provided in the calculations, but the exact results no longer hold.
- The distributions of the two samples are identical (although not necessarily normal) and differ only in location (i.e., central tendency).
- The two samples are independent.

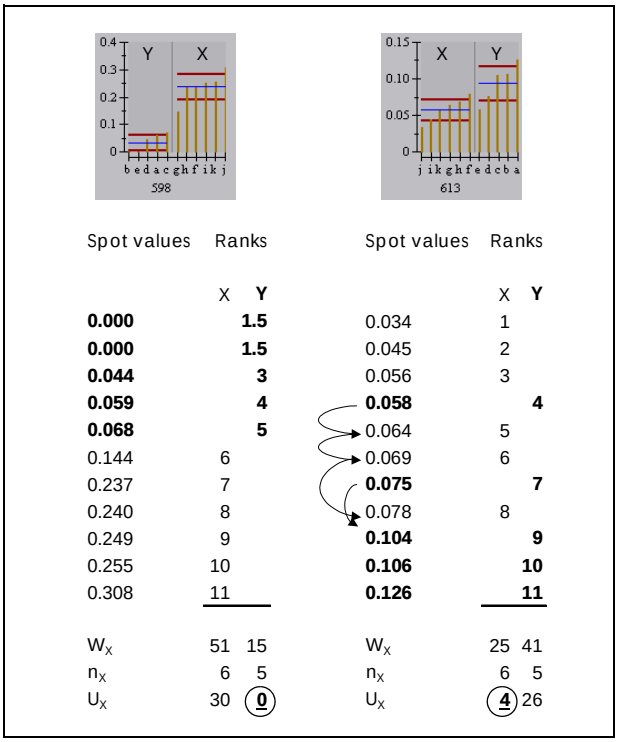
To perform the Mann-Whitney test, the software first ranks all the spot values from low to high, paying no attention to which of the two classes (e.g., X and Y) each value belongs. Then each value is given a rank number. The smallest number gets a rank of 1. The largest number gets a rank of N, where N is the total number of spot values in the two classes. If two values are the same, then they both get the average of the two ranks for which they tie. Finally, the ranks in each class are summed, thus giving WX and WY, which are used to calculate the Mann-Whitney test statistic, U. The formula for UX is as follows (the formula for UY is obtained by replacing X by Y):

$$U_X = W_X - \frac{n_X(n_X + 1)}{2}$$

The smaller of the two calculated U values corresponds to the number of shifts needed in order that the spot values from the two populations do not overlap. For the first example in Figure 8-15, no shifts are necessary since the spot values of classes X and Y are already separated. On the contrary, for the second example the Mann-Whitney test indicates that the first two values from Y (or the three last values from X) have to be swapped four times in order to separate the samples.

The software displays the smaller of the two calculated U values in the Class Analysis Table. The lower the number, the higher the probability is that the means of the two samples are different. Knowing this value, and the sample size, you can easily look up the probabilities in a Mann-Whitney table.

Caution should be used when analyzing the results of a Mann-Whitney test. First, the assumptions are often violated. This is the case when spots are completely absent in one of the classes (in this case you have repeated values of 0). Moreover, if you have small samples, the Mann-Whitney test is meaningless. If the total sample size is seven or less, the test always gives a probability (of finding different means, in the case of identical populations) greater than 0.05, no matter how little the samples differ.



Spot values	Ranks		Spot values	Ranks	
	X	**Y**		X	**Y**
0.000		**1.5**	0.034	1	
0.000		**1.5**	0.045	2	
0.044		**3**	0.056	3	
0.059		**4**	**0.058**		**4**
0.068		**5**	0.064	5	
0.144	6		0.069	6	
0.237	7		**0.075**		**7**
0.240	8		0.078	8	
0.249	9		**0.104**		**9**
0.255	10		**0.106**		**10**
0.308	11		**0.126**		**11**
W_X	51	15	W_X	25	41
n_X	6	5	n_X	6	5
U_X	30	**0**	U_X	**4**	26

Figure 8-15. Two examples to illustrate the Mann-Whitney and Kolmogorov-Smirnov tests. In (a) the two classes do not overlap at all, whereas in (b) 4 shifts are needed to completely separate the spot values of the two classes. The bold values correspond to the spot values and ranks from class Y, the others belong to class X.

Kolmogorov-Smirnov test

The Kolmogorov-Smirnov test tries to determine if two data sets differ significantly. It is used to test whether or not two samples may reasonably be assumed to come from the same distribution. It has the advantage of making no assumption about the distribution of the data and is frequently preferred over the Mann-Whitney rank sum test where there are many ties (repeated values). Please note that this generally comes at a price. Other tests (e.g., the t-test) may be more sensitive if the data meet the requirements needed for that test. The assumptions of the Kolmogorov-Smirnov test are:

- The probability distributions are continuous.
- The measurement scale is at least ordinal.
- The two samples are mutually independent.

In the Kolmogorov-Smirnov test, the data points in each sample (spot values for a particular match in a class) are sorted in ascending order

and converted into an empirical distribution function (EDF). This function gives the fraction of data points to the left of a given value z. For the second example in Figure 8-15, the ordered data points from class X are: 0.034, 0.045, 0.056, 0.064, 0.069, and 0.078. The fraction of data points to the left of each of these z values can easily be calculated and plotted (full line) in an Empirical Distribution Plot (Figure 8-16):

It is clear that no data lie strictly below 0.034, 17% = 0.17 = 1/6 of the data is strictly smaller than 0.045, 33% = 0.33 = 2/6 of the data is strictly smaller than 0.056, 50% = 0.50 = 3/6 of the data is strictly smaller than 0.064, and so on.

The same procedure can be followed for the second sample (class Y in our example, the dashed line in Figure 8-16). The Kolmogorov-Smirnov test statistic D is then defined as the maximum distance between the empirical distribution functions (EDF) for the two samples. In the example, D is 0.63 (0.83-0.20). If D is greater than a particular decision limit (critical value found in a Kolmogorov-Smirnov table), there is a statistically significant difference between the two samples. However, the test provides no insight as to what causes the difference.

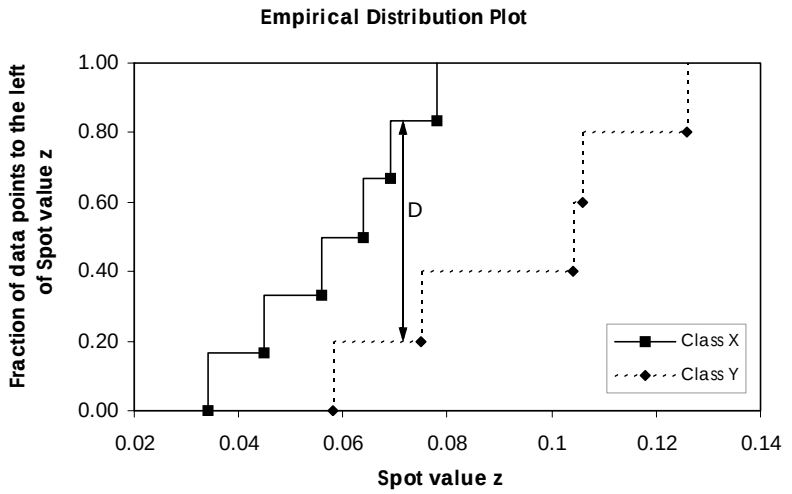
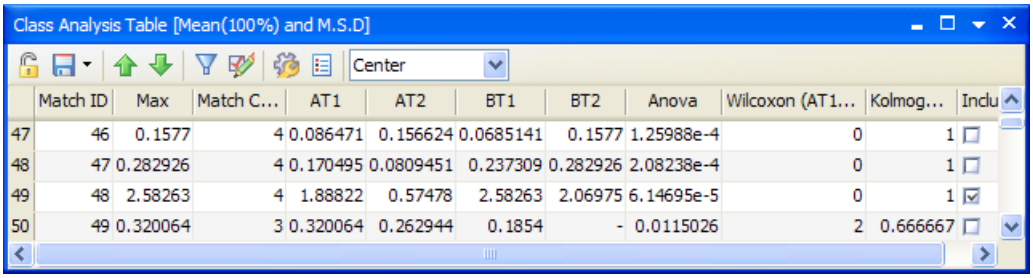


Figure 8-16. The Empirical Distribution Plot for the spot values of match 613 (Figure 8-15), in Classes X and Y. The Kolmogorov-Smirnov statistic D corresponds to the maximum distance between the two empirical distribution functions.

8.4.5 Class analysis table

The Class Analysis Table provides valuable data for finding significant protein expression changes between populations of gels. Using this data, it is possible to differentiate one class from the others based on just a few matches/spots. Choose ***Reports > Analyse Classes > Table*** to display the Class Analysis Table (Figure 8-17). For each match, there is a description of:

- The ***Center*** (central tendency), ***Dispersion***, ***Gap***, ***Ratio*** or ***Normalized*** values for each class, or fold change versus a selected reference class X (***versus Class X***) depending on the selection made in the drop-down list (see below). By default, the Center values are displayed.
- The ***Max*** value, that is, the highest from all these class values.
- The ***Match Count***, which is the number of classes in which the spot is present and matched.
- The results from the statistical tests: the ANOVA probability P, the Wilcoxon U statistic, and the Kolmogorov D statistic.
- The ***Fold***-change between the class with the lowest Center value and the class with the highest Center value.
- The columns corresponding to spot sets.



	Match ID	Max	Match C...	AT1	AT2	BT1	BT2	Anova	Wilcoxon (AT1...	Kolmog...	Inclu
47	46	0.1577	4	0.086471	0.156624	0.0685141	0.1577	1.25988e-4	0	1	☐
48	47	0.282926	4	0.170495	0.0809451	0.237309	0.282926	2.08238e-4	0	1	☐
49	48	2.58263	4	1.88822	0.57478	2.58263	2.06975	6.14695e-5	0	1	☑
50	49	0.320064	3	0.320064	0.262944	0.1854	-	0.0115026	2	0.666667	☐

Figure 8-17. Class Analysis Table.

In addition to the standard functionalities for saving, printing, copying content to the clipboard and navigating in the report, the following tools in the Class Analysis Table are particularly useful:

Select by value
Select items in the table based on a numerical search criterion. Choose the measure (i.e., column) to be used for refinement, and set the lower and/or upper limits of your search interval.

Create spot set
Create a spot set to annotate spots of interest for use at a later stage. All currently selected spots are automatically included in the newly created spot set, which appears as a column in the table. You see a checked box for spots that belong to the set, or an empty box for spots that do not belong to the set.

Statistics
Set the statistics to be used for calculating the Center and Dispersion value of each class, and consequently the Gap, Ratio and Normalized

values. These settings are common to the Class Analysis Table and Class Analysis Histograms.

CAUTION! The Center and Dispersion values define the interval that characterizes the protein sample of each class in a match. To characterize a class only by the central value, set the Dispersion percentage slider to 0%. This is useful if you want to calculate the difference or ratio between the central values of your classes. With the Mean (100%) selected as Central Tendency and the Dispersion slider set to 0%, the Gap and Ratio values that are calculated in the Class Analysis Table correspond to the classical way to calculate the difference or ratio between populations.

Alternatively, you can use the Fold and versus Class X options (see 8.4.3 Fold change).

Settings

Some of the above-mentioned columns may not be displayed by default. Click the ***Settings*** icon to show or hide columns from the table.

Displayed value

The displayed statistical descriptor or overlapping measure can be selected from the drop-down list in the toolbar. You can display the Center (central tendency), Dispersion, Gap, Ratio or Normalized values for the different classes, as well as the ratio versus a selected reference class (versus Class X).

CAUTION! *The Gap, Ratio and Normalized options are not available for DIGE experiments when Vol Ratio is selected as the quantification measure.*

8.4.6 Class analysis histograms

You can visually investigate the statistical and overlapping descriptors of populations by displaying Class Analysis Histograms. Just choose ***Reports > Analyse Classes > Histograms***.

When the Center (central tendency) value is selected for display in the drop-down list, the Class Analysis Histograms display all the individual spot values in each match, separated for each class by vertical gray lines (Figure 8-18). The classes are characterized by their central tendency (blue horizontal line) and dispersion interval (bounded by the outer red lines). The Match IDs appear below each histogram.

When displaying the Dispersion, Gap, Ratio or Normalized values (Figure 8-19), each class is represented by a single value (red bar).

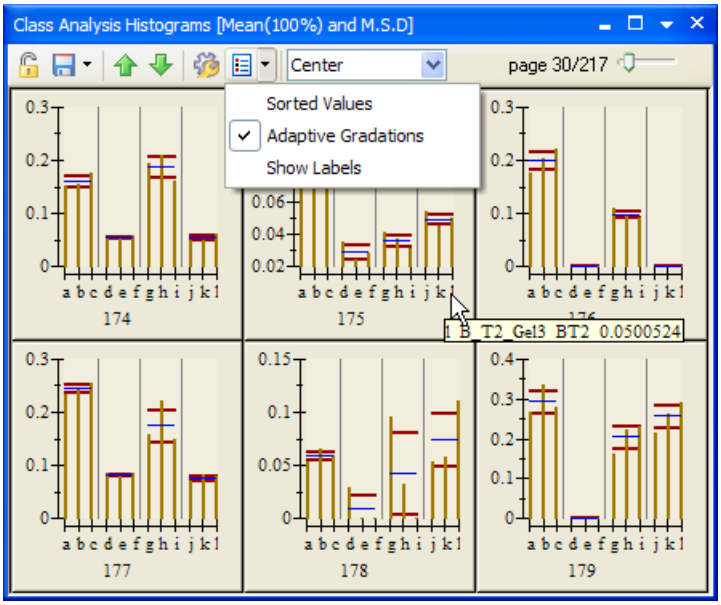


Figure 8-18. Class Analysis Histograms with Center values displayed.

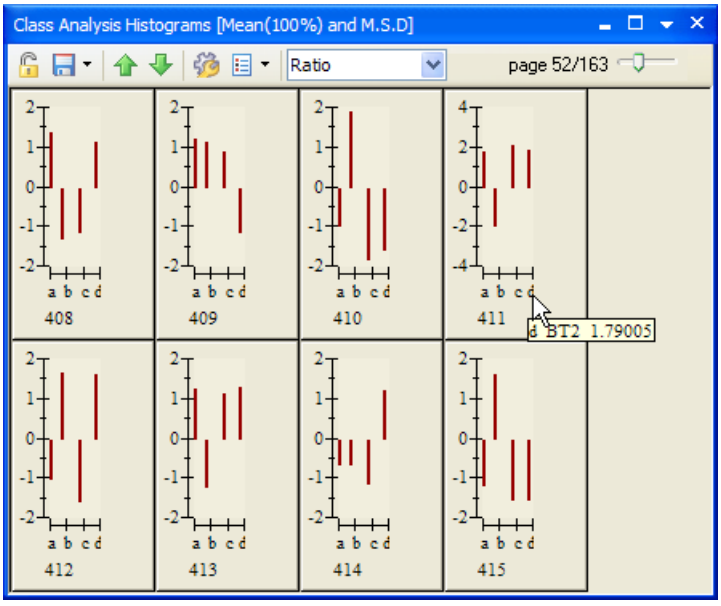


Figure 8-19. Class Analysis Histograms with Ratio displayed.

The histograms can be selected to highlight the corresponding spots on the gels and in any open reports.

The following tools in the Class Analysis Histograms are very useful:

Slider

To see the matches displayed on additional pages, use the slider in the toolbar. When a match is selected on an image or in another report, it is automatically highlighted in a histograms window.

Statistics

Set the statistics to be used for calculating the Center and Dispersion value of each class, and consequently the Gap, Ratio and Normalized values. These settings are common to the Class Analysis Table and Class Analysis Histograms.

Settings

Click the ***Settings*** icon to activate one of the following options:

- ***Sorted Values***, to sort the classes according to their central value and sort the spot values inside each class (Figure 8-20). This can simplify the search for non-overlapping intervals.

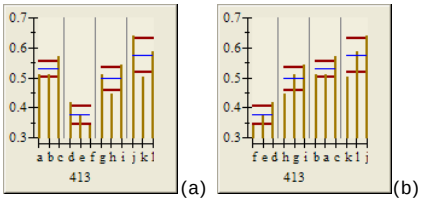


Figure 8-20. Class Analysis Histograms with (a) unsorted values and (b) values sorted in ascending order.

- ***Adaptive Gradations***, to adjust the histogram gradations according to the values in each histogram. Deselect this option to display an identical gradation in all histograms (Figure 8-21).

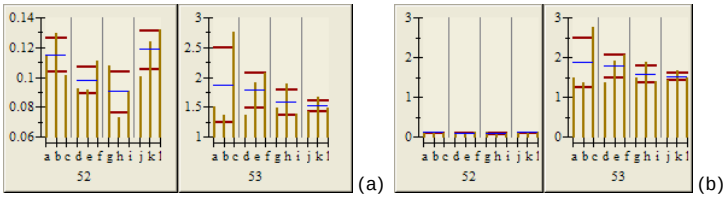


Figure 8-21. Class Analysis Histograms with adaptive gradations set (a) individually for each histogram and (b) according to the minimum and maximum values in all histograms.

- ***Show Labels***, to display a table with the gel or class names. Alternatively, when you place the mouse over a letter in the

histograms, a screentip displays the full image or class name and the value in that image or class.

Displayed value

The displayed statistical descriptor or overlapping measure needs to be selected in the drop-down list in the toolbar. You can display the Center (central tendency), Dispersion, Gap, Ratio or Normalized values for the different classes.

CAUTION! *The Gap, Ratio and Normalized options are not available for DIGE experiments when Vol Ratio is selected as the quantification measure.*

9 Annotations

9.1 Introduction

Individual pixels and spots in a gel image can be labeled with annotations. Annotations are used to flag items with their specific characteristics (protein name, database accession number and so on) or to mark spots with common characteristics. They also offer the possibility of linking and associating gel objects to external query engines or data sources of any format (text, html, spreadsheet, multimedia, database) located locally or on the Internet.

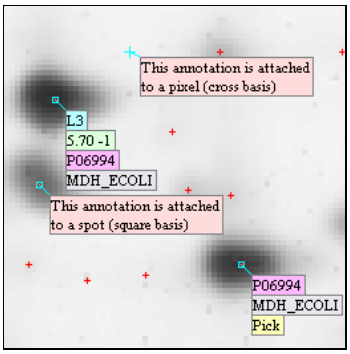


Figure 9-1. Annotations are composed of an annotation basis (square or cross), an annotation flagpole and a set of labels (flag).

An annotation is defined by its position on the gel (X and Y coordinates) and its set of labels. Each label belongs to a predefined or user-defined category. As shown in Figure 9-1, each annotation is composed of a basis, which can be a square or a cross depending on whether the annotation is attached to a spot or a pixel, a flagpole, and a flag that consists of a set of colored labels.

Spot and pixel annotations

You can create two types of annotations (Figure 9-1):

- **Annotations linked to pixels** are visualized with a **cross** basis. They are simply connected to a pixel and have the same coordinates as that pixel.
- **Annotations linked to spots** are visualized with a **square** basis. They are linked to a spot and have the same coordinates as that spot, that is, as the spot's center of gravity. These annotations are automatically selected when the linked spot is selected and vice versa.

9.2 Create annotations and labels

9.2.1 Create annotations

The creation of annotations essentially consists of taking three steps.

1 Select the spots or pixel on which you would like to create an annotation:
   - Choose the ***Select*** tool and double-click on a spot or a pixel.
   - Select several spots, make sure to select only the images on which you want to create new annotations, and choose ***Edit > Annotations > Add***.

2 Choose one of the existing categories or create a new category. The creation of new categories is described in Section 9.3.

3 Enter the desired label content.

9.2.2 Add labels to existing annotations

Labels can be added to an existing annotation. The procedure is almost identical to that described above, with some slight differences in the first step.

1 Select the annotation to which you would like to add a label:
   - Choose the ***Select*** tool and double-click at the basis of the annotation to which your label should be added.
   - Select several annotations, make sure to select only the images on which you want to create new labels, and choose ***Edit > Annotations > Labels > Add***.

2 Choose one of the existing categories or create a new category. The creation of new categories is described in Section 9.3.

3 Enter the desired label content.

Note: *One annotation may have many labels, but it can only contain one label from each category. If one spot contains several proteins, it may need to carry different labels from the same category. You can do this by linking additional annotations to the spot.*

9.2.3 Link annotations to spots

The software allows you to link an annotation to a spot. This is helpful when you want to link an additional annotation to a spot (to attach multiple labels of the same category), or when you missed a spot to which you wanted to link a newly created annotation. You may also decide that, finally, an annotation should not be linked to a given spot.

To link an annotation with a spot, click on the annotation basis and drag it to a spot. If, for some reason, an annotation already exists within a spot

but is not yet linked, you can also select the annotation and choose ***Edit > Annotations > Link with Spot***.

To unlink an annotation from a spot, select the annotation and drag it outside the spot or choose ***Edit > Annotations > Unlink from Spot***.

9.3 Create label categories

9.3.1 Predefined label categories

The software offers some predefined label categories:

- **Ac**: This category is provided to hold the protein's accession number (AC) taken from a user-defined database and can be the linke to Melanie's remote database query engine. When such a link is defined, double-clicking on a label of this category displays the corresponding protein entry in the selected database with the default Internet browser.
- **pI_MW**: This category contains the known isoelectric point (pI) and molecular weight (MW) information, which is subsequently used to compute approximate pI and MW values for any point in a gel. You should enter the pI value first and MW value second, separated by a space. By replacing one of the values by -1, you indicate to the program that no value is set (probably because you do not know it).
- **Comment**: This category is an example of a general category where users may store their comments.

9.3.2 Create new categories

When you create a new category during one of the procedures described in Section 9.2, you must enter the constraints and attributes for the new category in the Create Category window (Figure 9-2). This section explains the different options.

> **CAUTION!** User-defined categories are only available from the category list as long as there is at least one label of this category in the open gels (in any of the open sheets). To create categories that are permanently available, you must define them in the ***Annotations*** tab of the ***Options*** window (available by choosing ***Tools > Options***). The same category constraints must be defined.

At any moment, you can change the category constraints by choosing ***Edit > Annotations > Categories > Edit Attibutes***, or rename a category by choosing ***Edit > Annotations > Categories > Rename a Category***.

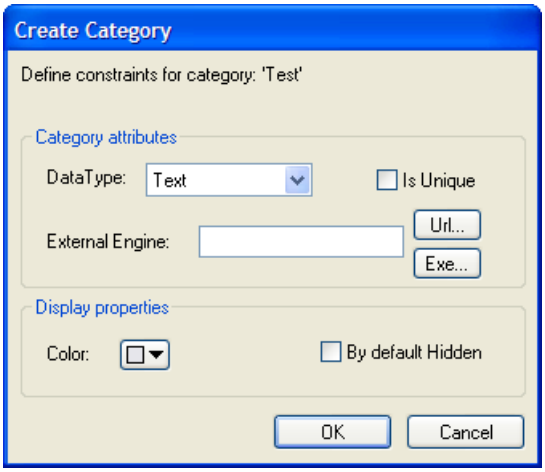


Figure 9-2. Create Category window.

Data type

By default, the labels can contain any character. However, to ensure coherent annotation data, the label contents can be constrained to one of the following ***Data Types***:

- ***Text***. Can contain any character.
- ***Number***: Can only contain numerical values.
- ***Auto-Numbering***. An incremental number (per gel) is entered automatically as the new label.
- ***Set***. Use this data type to mark spots with common properties. The labels in such a category do not contain specific information. They only display the name of the category to which they belong. Note that labels of this category are displayed in the form of check boxes in tables. A checked box indicates that the spot belongs to the category.

Is unique

When you check the ***Is Unique*** box, you indicate that each label on a gel within the new category should be unique. The software will not accept a new label when an identical one already exists. You are asked to enter a new label.

External engine

Melanie offers the possibility to link spots on gel images to protein data in 2-DE or other databases. All you have to do is input the appropriate query format (database address and query engine) in the ***External Engine*** field of the new label category and enter valid database accession numbers (AC) as labels. This functionality is further described in Section 9.4.

When you subsequently double-click on a label of such a category, the software opens your default Internet browser and launches an HTTP query that takes the form of a Web page address.

Please note that you can define a different query engine for each label category and therefore you can link one protein spot to different database entries.

Display properties

User-defined categories use a gray background color by default. You can change the default color by clicking on the ***Color*** box. The new background color is used for all labels of that specific category.

It is often not desirable to have all annotation categories displayed, because this can clutter the display. You can therefore choose to hide the category by default, by clicking the ***By default hidden*** box. In this case, you must choose ***View > Annotations > Visible Categories*** to make the category visible by checking its box.

9.4 Connect to protein databases

9.4.1 Introduction

Melanie can link spots on gel images to protein data in 2-DE or other databases. Such databases contain information on proteins identified on 2-DE images, such as pI and MW values, bibliographical references to protein related literature, information on protein functions, etc. If your computer has Internet access, you can remotely query and retrieve protein data related to spots on your gels.

CAUTION! The Melanie software provides access to several databases on the Internet. It is the responsibility of the user to acquire the database licenses, if needed. In particular, the PROSITE and SWISS-2DPAGE databases are copyrighted, and all commercial users of these databases are required to purchase a database license from GeneBio. No license fee is charged to academic users for non-commercial use. For questions about obtaining a license subscription for the PROSITE and SWISS-2DPAGE databases, please contact GeneBio (www.genebio.com).

Melanie takes advantage of the fact that virtually all databases on the Web, and in particular those containing 2-DE and other protein data, use CGI scripts to enable data queries. A CGI (Common Gateway Interface) script is a program or script file executed on a Web server in response to a user request. The CGI script transmits information (such as a database accession number or object identifier) from the client to a database engine, receives back the results, and displays them to the client.

9.4.2 Set the database

To query a remote database through the Internet, you must send HTTP queries (for instance, http://www.expasy.org/swiss-2dpage/P02649) to a CGI script on a server. The HTTP query must be composed of:

- The database HTTP address (http://www.expasy.org/).
- The database query engine (swiss-2dpage/).
- The database accession number (P02649).

In Melanie, the database HTTP address and query engine are entered as constraints to the annotation category (see Figure 9-3). Type them in as one string in the ***External Engine*** field (for example, http://www.expasy.org/swiss-2dpage/).

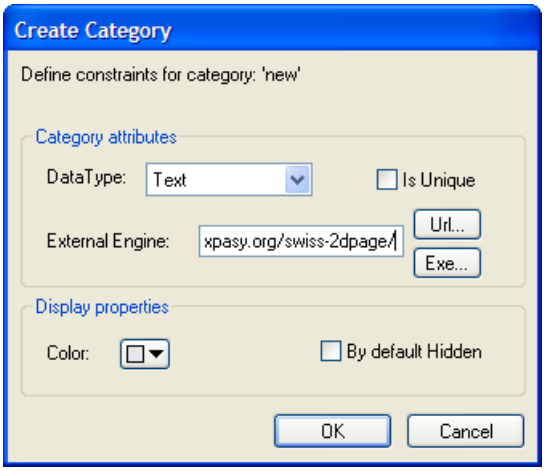


Figure 9-3. Setting the External Engine as a category constraint.

Federated 2-D PAGE databases

A list of federated 2-D PAGE databases, with the required database query formats, can be found at http://www.expasy.org/ch2d/2d-access.html, or by clicking on the ***Url*** button to the right of the External Engine field. Copy the desired database address and query engine from this site.

Other databases

It is possible to find the required query format for databases that are not federated or that do not contain 2-DE data. Directly query the database until you find a specific protein (or other) entry. Then copy the address of the corresponding Web page in your browser to the External Engine field of the Create Category window, without including the accession or identification number of the current entry. Generally, this address consists of the database HTTP address and query engine followed by a question mark (?) or equal sign (=).

For example, you might display the entry for the protein structure 1BMT in the well-known Protein Data Bank (PDB): http://www.rcsb.org/pdb/explore/explore.do?structureId=1BMT. The required query format for Melanie is: http://www.rcsb.org/pdb/explore/explore.do?structureId=

9.4.3 Query the database

The database accession number (for example P02649) is entered as a label of the particular category linked to a spot.

When you subsequently double-click on the label while the ***Selection*** tool is selected, Melanie opens your default Internet browser and launches an HTTP query that takes the form of a Web page address.

As a result, the entry for the protein with the given accession number opens in your browser (Figure 9-4). In the case of the above example, this would be the entry for Human Apo E (Gels) in the SWISS-2DPAGE database.

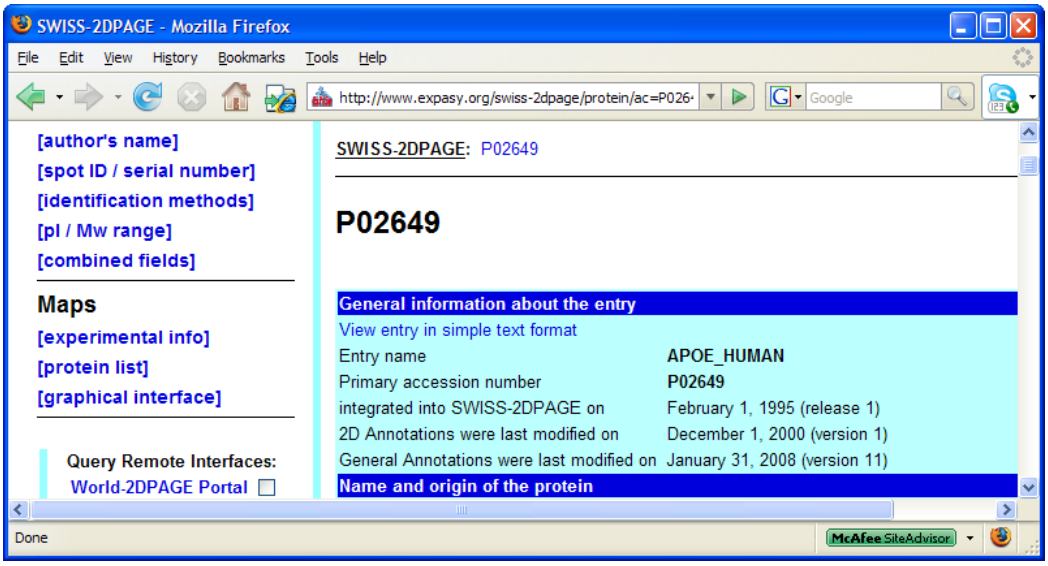


Figure 9-4. The SWISS-2DPAGE entry for Human Apo E. Entries from this database contain full protein names, bibliographic references, annotations (such as protein function or pathological variations), and the pI and MW of the related spots on the 2-DE maps. It also includes cross-references to numerous other databases.

9.4.4 Connext to an executable

Analogous to an HTTP query, where the content of a label is transmitted to a CGI script on a server, Melanie allows you to pass on the content of a label as the first parameter to any executable. When you double-click on a label that has an executable defined in the ***External Engine***

field of the label category, the executable runs with the label content as a parameter.

To define an executable in the External Engine field, click on the ***Exe*** button and locate the .exe file.

9.5 Create specific links

As seen above, it is possible to link labels to remote database entries by defining an external search engine for a particular category. By using specific keywords in the labels of any category, you can also create links to Web pages, files, and text (Figure 9-5).

To create a specific link, you should add an annotation to a gel and include the following items in the label field:

- A **short descriptor** that will be the visible part of the label.
- A **keyword** indicating the type of link (http:, file:, text:).
- A **link content**, which contains the information necessary to establish the link or the content of the link (in the case of a text link).

To indicate that a label is linked, its short descriptor is followed by three dots. When you double-click on such a linked label, you do not enter the typical editing mode, but the link (Web page, file or text) is automatically opened with the appropriate software. Alternatively, to open any linked label, choose ***View > Annotations > Linked Data*** in the menu.

Note that you can define links in any label category but you can only have one link per label.

9.5.1 Http link:

You can link spots or pixels to specific Web pages. A double-click on an http-linked label will launch your Internet browser and automatically call the corresponding Web page.

You can, for example, create a direct link to the ExPASy Proteomics Server (Figure 9-5). In this case, the label content should contain the string “http:” followed by the address of the Web page.

9.5.2 File link:

You can link spots or pixels to software files. Double-clicking on a file-linked label launches the specified file with the default system application associated to the file extension.

The linked files can be placed in a specific directory, which is defined by choosing ***Tools > Options*** in the menu and by setting the ***Annotations*** folder in the ***Annotations*** tab. In this case, you only need to give the name and file extension when creating the link. You can create subfolders in the Annotation folder to arrange your files. The file names

indicated in the labels must then contain the name of the subfolder (e.g., AA composition\AA P10413.xls).

Alternatively, you can link labels with files located anywhere on your system. You should then include the complete file path when referencing the file.

For example, you can link a protein spot to an Excel file containing the amino acid composition of a protein (Figure 9-5). The label should then consist of the string “file:” followed by the file name (with its extension).

9.5.3 Text link:

In some cases, you might want to associate a long text comment with a specific protein spot, but without overloading the display. The solution is to create a text link, rather than a very long annotation label. Double-clicking on the linked label is sufficient to display a window containing the entire text (Figure 9-5).

Text links are particularly useful for attaching bibliographic references to a spot, for instance, or any other comment such as the one in Figure 9-5. Please note that the string "text:" must first be inserted, followed by the text you would like to associate with the spot.

To connect general information about the gel, other tools are better adapted. Comments can be attached to projects, match sets, and classes in the workspace. Additionally, you can specify gel descriptions.

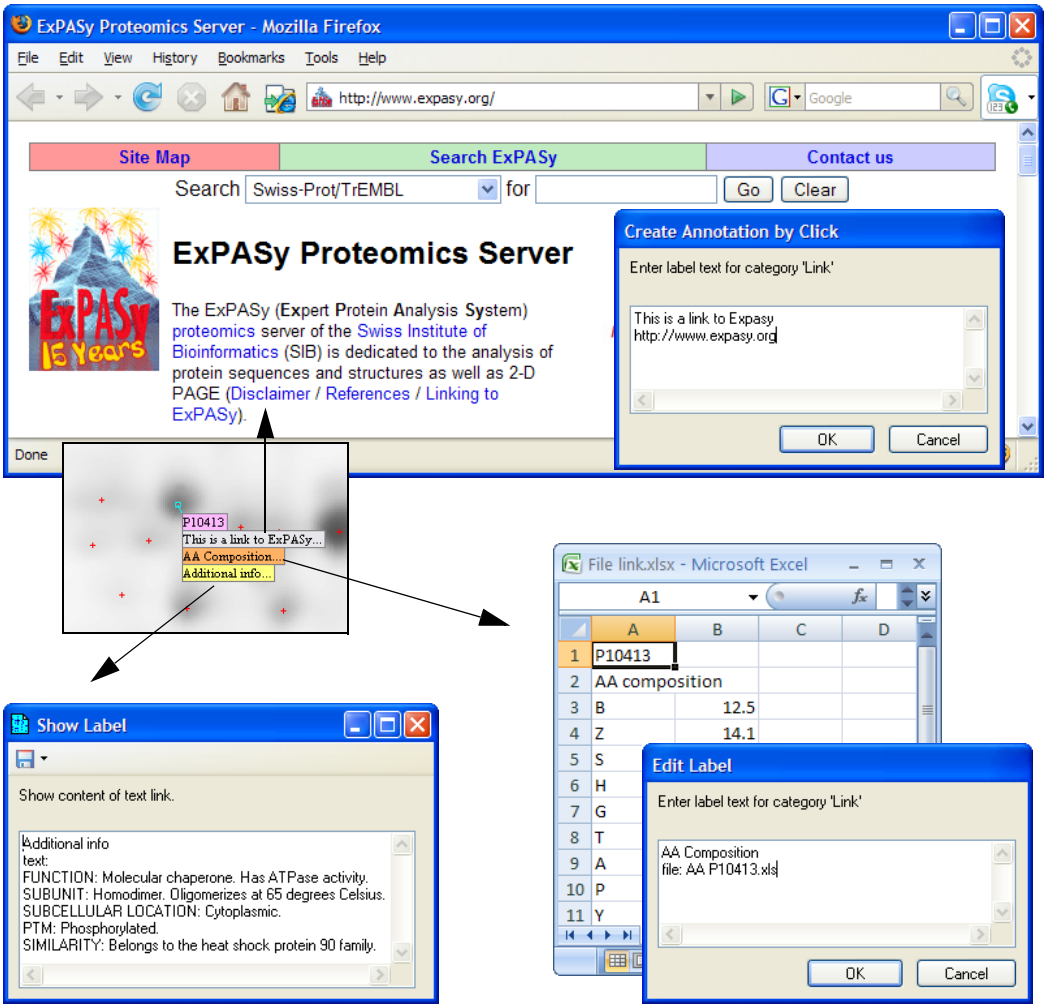


Figure 9-5. The annotation on the spot containing protein P10413 includes linked labels such as a link to a Web site (top), a link to a file (bottom right) and a text link (bottom left).

9.6 Select annotations and labels

9.6.1 Select

Annotations and labels can be selected with the ***Select*** tool. The selected labels or annotations are highlighted in green and displayed in front of the other annotations (Figure 9-6).

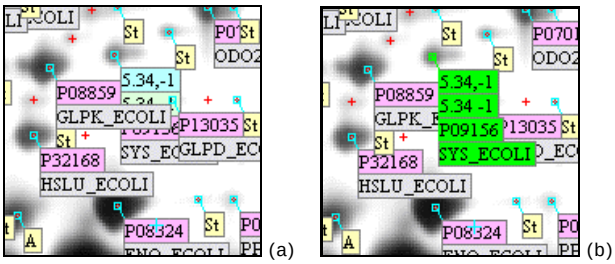


Figure 9-6. (a) Annotation hidden by some other annotations. (b) Same annotation displayed in front of all other annotations, after selection.

If the annotation is attached to a spot, the spot is also selected. Similarly, if you select a spot, the attached annotation is selected.

To select a label, click on the label. You can select more than one label by using the Shift key.

To select an annotation, click on the annotation basis. To select more than one annotation, select the first one, then hold down the Shift key and select additional annotations.

To select all annotations in a region, position the cursor at the top left position of the desired region, hold down the left mouse button, and then drag the cursor to the bottom right position. All annotations in the defined region are selected. To select annotations in more than one region, hold down the Shift key while selecting additional regions.

To deselect all annotations, click on a gel (not on an annotation).

9.6.2 Select menu

You can select specific labels and annotations with the options in the ***Select > Annotations*** menu. Please note that this also selects hidden annotations.

- ***By Content***: This feature enables the selection of labels (belonging to one or several categories that must be selected) based on their content. When the ***Regular Expression*** box in the window is not checked, the entered string of characters is taken literally, and the program selects all labels containing this string. By activating the Regular Expression option, regular expressions can be used in the search field (see below for details). For example, to select all labels containing the string P00, you can either type the expression .*P00.* and choose the Regular Expression field in the window, or type P00 and deselect the Regular Expression field.
- ***By Category***: This feature enables the selection of all labels belonging to one or several categories. Use the Shift and Ctrl keys to pick several category names at a time.
- ***All***: This highlights all annotations in the selected gels.

- ***Common Labels***: This option allows the retrieval of all sets of identical labels within a gel or among a series of gels, for all the categories chosen in the window.

Regular expressions

Regular expressions provide a mechanism to select specific strings from a set of character strings. Regular expressions use symbols and syntax elements to describe a generalized pattern. Melanie invokes the standard Extended Regular Expressions to search patterns in labels, the essentials of which are summarized in Table 9-1 .

Syntax	Description	Example
.	Matches any one character.	**e.oli** matches:eaoli, eboli, ecoli...
[...]	Matches any character listed between the brackets. [a-z] indicates the range of characters between A and Z and [0-9] is any numeral from 0 to 9.	**P[a-d]** matches:Pa, Pb, Pc, Pd
[^...]	Matches any character except those listed between the brackets.	**P[^bd]** matches:Pa, Pc, Pe but not Pb or Pd
?	Matches the preceding element zero or one times.	**P0?1** matches:P1 and P01.
+	Matches the preceding element one or more times.	**P0+1** matches:P01, P001, P0001, ...
*	Matches the preceding element zero or more times.	**P0*1** matches:P1, P01, P001, P0001, ...
{n}	Matches the preceding element exactly n times.	**P0{3}1** matches:P0001 but not P01 or P001
{n,}	Matches the preceding element at least n times.	**P0{2,}1** matches:P001, P0001, ... but not P01
{n,m}	Matches the preceding element at least n times, but not more than m times.	**P0{1,3}1** matches:P01 P001, and P0001, but not P1 or P00001
()	The characters between parentheses form a subexpression.	**P(24)+** matches:P24, P2424, P242424, ...

Syntax	Description	Example
|	Matches the expression before or after the vertical line. Mostly used within a subexpression.	**P(ab|cd)1** matches:Pab1 and Pcd1
^	A circumflex outside a bracket expression anchors the element it starts with to the beginning of a string; such an element can match only a sequence starting at the first character of a string.	**^(ec).*** matches:ecoli, ecoli_eftu but not eftu_ecoli
$	A dollar sign outside a bracket expression anchors the element it terminates with to the end of a string; such an element can match only a sequence ending at the last character of a string.	**.*(ecoli)$** matches:ecoli, eftu_ecoli but not ecoli_eftu
.	Matches any one character.	**e.oli** matches:eaoli, eboli, ecoli...

Table 9-1. Regular expressions available to search patterns in labels. Please note that the term element used in the description indicates a character or a subexpression.

The characters ^.[$()|*+?{\ have a special meaning in certain contexts. You must exclude them from the expression with a backslash (\) if you want them to be taken literally. This means that if your labels contain any of these special characters, you must precede them with a backslash if you want to include them as normal characters in your search expression. Note that you must also release the backslash character itself from the expression. For example, the search pattern R*.* returns the result R*3.24 but not R/2.87.

Nevertheless, bracketed expressions are an exception to the rule. Inside bracketed expressions, all special characters, including the backslash, lose their special meaning (for instance, [*\+?{}.] matches exactly any of the characters inside the brackets).

The order of precedence for the regular expressions described above is as shown in Table 9-2 . For example, the regular expression abc2|3de matches either the string abc2 or the string 3de (rather than the string abc2de or abc3de) because concatenation has a higher order of precedence than alternation.

Escaped characters	\<Special character>
Bracket expression	[ ]
Grouping	( )
Single-character duplication	* + ? {m,n}
Concatenation	
Anchoring	^ $
Alternation	|

Table 9-2. Order of precedence (from high to low) for regular expressions.

9.6.3 Reports

You can select annotations by selecting their corresponding lines in the Annotation Table, available by choosing ***Reports > Annotation Table***.

9.7 Display annotations and labels

You can change the way annotations and labels are displayed.

9.7.1 Annotation flag position

Sometimes you may want to move an annotation flag because you are preparing an illustration or just want to see what lies underneath. To interactively change an annotation's flag position, click on one of the labels and drag the flag to the desired position (Figure 9-7).

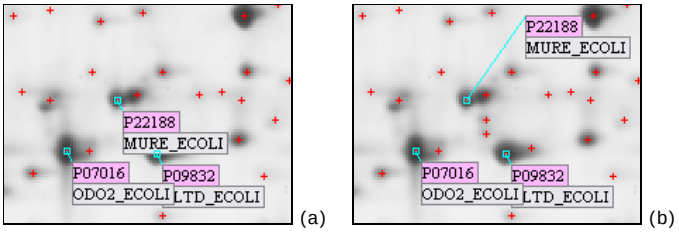


Figure 9-7. (a) Default and (b) modified annotation flag position.

9.7.2 Visibility of annotations and labels

You can quickly cover entire gel images with a considerable number of annotations and labels, which are not always necessary at any given moment in the analysis. Therefore, you can hide all annotations or

certain label categories in selected gels. These options are available from the ***View > Annotations*** menu:

- ***Visible categories***: Sets the visibility state of the various categories on the selected gels. To hide a category, make sure its box is unchecked. On the other hand, select an empty check box to show the corresponding category. You can click once or twice in a grayed check box (category that takes different visibility states in the various selected images) to hide or show the corresponding label category in the selected gels.
- ***Show All***: Makes all the annotations on the selected gels visible, including the labels that were hidden.
- ***Hide All***: Hides all the annotations on the selected gels, including the cyan cross or square that remains visible when all labels, but not annotations, are hidden.

When you click on an annotation that has hidden labels, all of its labels are displayed on the screen during the time it remains selected (Figure 9-8). The hidden labels disappear when the annotation is deselected.

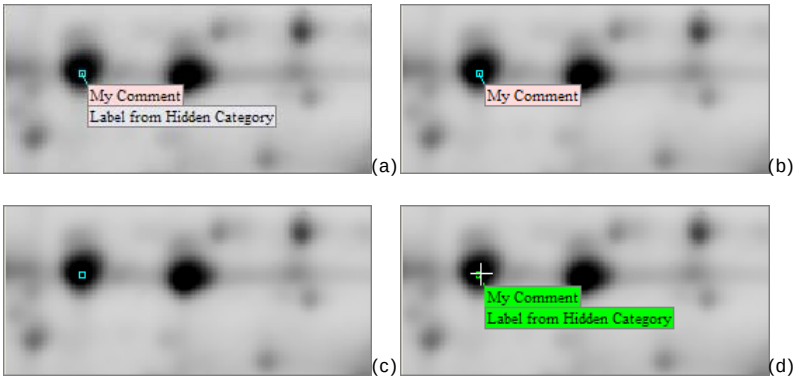


Figure 9-8. (a) Unselected annotation with two labels. (b) Unselected annotation with 1 label hidden. (c) Unselected annotation with all labels hidden. (d) When the annotation is selected the hidden labels become visible.

9.8 Edit annotations and labels

You can add and modify annotations and labels in different ways. The ***Select*** tool is helpful if you want to add or modify a single label or just a few labels. The menu options are more adapted to the creation of a large number of annotations or labels simultaneously. Reports are useful for editing existing annotations.

9.8.1 Select

When double-clicking on a label while the ***Select*** tool is activated, the Edit Label window is displayed. Change the text in this box to modify your label content.

The ***Select*** tool should also be used to change the position of an annotation. In this case, simply select an annotation and drag its basis to the new location.

9.8.2 Edit menu

Various options for editing labels or annotations are available from the ***Edit > Annotations*** menu. All of these features can be used to edit several labels or annotations at a time.

Delete annotations and labels

Two possibilities are available for deleting selected labels or annotations:

- ***Edit > Annotations > Delete*** deletes the selected annotations.
- ***Edit > Annotations > Labels > Delete*** deletes the selected labels.

To delete labels from a specific category only, first select all labels from the desired category with ***Select > Annotations > By Category***.

Edit labels

You can change the content of selected labels (belonging to a single category) by choosing ***Edit > Annotations > Labels > Delete*** and entering the desired modifications in the Edit Labels window.

Copy / paste annotations

You can select annotations in a given gel image and copy them to the corresponding locations in other gels. This is a simple means of creating a set of similar annotations in a series of gels. Subsequently, you may need to adjust the annotation positions.

Select annotations on one image and choose ***Edit > Annotations > Labels > Copy***. Then select the gels into which you want to paste the annotations and choose ***Edit > Annotations > Labels > Paste***. Adjust the annotation positions using the ***Select*** tool.

Copy / paste labels

Instead of copying entire annotations, you can also copy distinct labels from one gel to selected spots or annotations in a series of gels.

Select the labels you would like to copy and choose ***Edit > Annotations > Labels > Copy***. Then select spots or existing annotations into which you want to paste the labels and choose ***Edit > Annotations > Labels > Paste***.

Propagate to matched

When gels have been matched, labels selected in one gel can be copied to their corresponding spots in other gels by choosing ***Edit > Annotations > Labels > Propagate to Matched***. This is particularly useful

when you have annotated one image during analysis, and want to propagate the labels to all matched gels.

Duplicate labels
You can copy selected labels to another category by choosing ***Edit > Annotations > Labels > Duplicate***. Since the selected labels may belong to different categories, this option can be used to merge several categories into a new one. However, only one label per annotation can be duplicated at a time.

9.8.3 Annotation table

You can edit labels via the Annotation table, available from ***Reports > Annotation*** Table. Make sure you have displayed the desired categories for editing, by clicking the ***Settings*** icon.

Create labels and label categories
Click on the ***Add Label*** icon in the Annotation Table to create new labels for selected spots. During this process, you can create a new label category, which will be inserted as an extra column in the table.

Add or modify labels
You can directly add new labels to the appropriate cells of the Annotation Table, or edit existing ones. Double-click in a cell to start typing your label, or modifying an existing label. When finished, a single click in any cell quits the editing mode.

Please note that categories using the Data Type Set are displayed in the form of check boxes in the table. These boxes show whether the corresponding item belongs to the category (checked box) or not.

9.8.4 Import annotations

You can import annotations from a file into open gels. Choose ***Edit > Annotations > Import*** to import annotations from an Annotation Report or a tab-delimited text file containing the required columns: SpotID, X, Y, and a column for each category to be imported.

If the Spot ID is not known, use -1 in this field (or remove this column) and the software will position the label in the corresponding X and Y positions of the gel. If X and Y positions are not known, use -1 in these fields and the software will position the label on the spot with the corresponding Spot ID.

9.9 Annotation table

The Annotation Table (Figure 9-9), availble by choosing ***Reports > Annotation Table*** provides specific information about annotations, and consequently labels and categories:

- Name of the image on which the annotation was created.

- SpotID, if the annotation is attached to a spot.
- Coordinates of the annotation.
- Calculated pI and MW values, if pI_MW annotations were defined on the image, or a matched image.
- A column for each label category, with the label content in the corresponding cells.

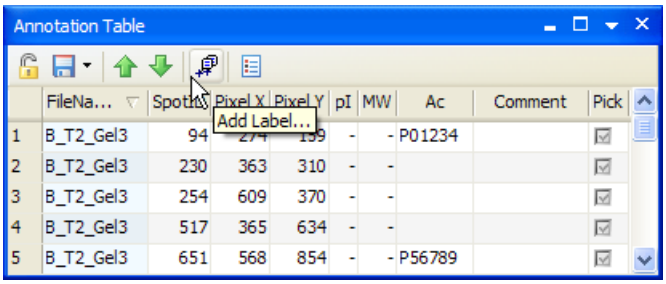


Figure 9-9. Annotation Table.

10 Data integration

10.1 Convert projects from earlier software versions

Melanie can Convert Projects created with versions 4, 5 and 6 of Melanie and ImageMaster 2D Platinum. Images analyzed with older versions must be added to a project in order to be imported into this new version and for spots, annotations and match data to be recovered. You can do this batch conversion for many projects at a time.

Choose ***File > Import > ImageMaster 2D Platinum or Melanie Data***, and indicate the folder where some or all of your projects (.prj files) are saved. The software searches and displays all project files found in this folder, and allows you to select projects for conversion (Figure 10-1). Then you must indicate the location where the converted projects should be saved. After conversion, the projects are automatically inserted in the workspace.

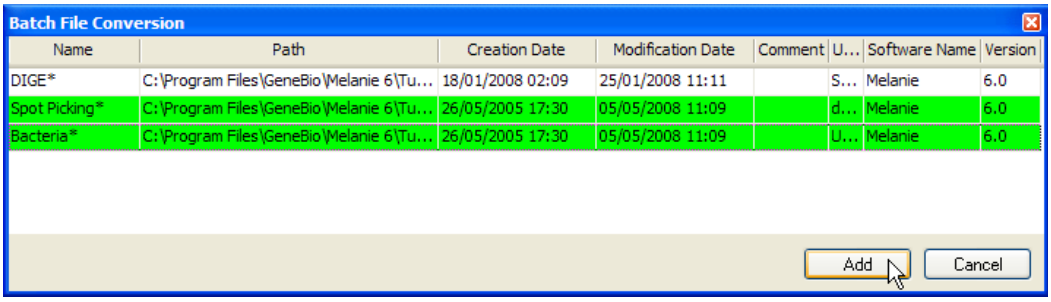


Figure 10-1. Select projects from previous Melanie or ImageMaster versions for Batch File Conversion.

10.2 Acquire images from Twain compatible scanners

Melanie can also acquire images directly from TWAIN compatible scanners.

10.2.1 Select source

You must indicate the scanning source by choosing ***File > Import > Twain > Select Source***. All TWAIN compatible scanners attached to your PC are automatically recognized by Melanie. This selection only needs to be done once (unless you want to change to a new image capture device).

10.2.2 Acquire

Launch the scan with ***File > Import > Twain > Acquire***. The scanner software opens, giving you the opportunity to change the necessary settings, and subsequently initiate the scanning process. Once this is done, the image is saved in Melanie file format and added to the Image Pool.

10.3 Export data

10.3.1 Reports

Data obtained in the analysis can be exported for use in other applications by saving the various tabular reports. Tables can be saved in text format (.txt), as a Microsoft Excel Workbook (.xls), or in XML (.xml) format. See below for more details on XML.

Graphical reports can be saved in PNG, TIFF or BMP formats.

10.3.2 XML

XML stands for eXtensible Markup Language and was created as a cross-platform, software, and hardware independent tool to structure, store, and exchange information. It allows the creation of customized tags, enabling the definition, transmission, validation, and interpretation of data between applications and organizations.

XML files can be viewed in the latest versions of Web browsers such as Internet Explorer, Netscape and Mozilla Firefox. However, as XML was designed to describe data and not to display data, it does not look like a Web page. An XML document contains color-coded root and child elements. A plus (+) or minus (-) sign to the left of the elements can be clicked to expand or collapse the element structure. If you want to view the raw XML source, you must select ***View > Source*** from the browser menu.

XML does not use predefined tags, as is the case for HTML. Therefore, the browser does not understand the meaning of the tags and does not know how to display the XML document. Therefore, XSL (eXtensible Stylesheet Language) stylesheets must be used in addition to the XML document to transform the XML into the sort of document that is recognized by the browser. This is the case when tabular reports or the history are printed from Melanie. The software uses the XSL stylesheets located in the Template folder of the Melanie installation directory to print attractive documents. If you are familiar with XML and XSL, you can even create personalized templates for printing. Note that XSL stylesheets can also be used to convert an XML file into another XML file.

The main interest in XML format is that external applications can easily extract necessary data. Moreover, the files can be converted to other user-defined formats.

CAUTION! Because the XSL stylesheets are specific to the browser you use, you will find that different versions (both for printing reports and the history) are installed with the software (in the Template\Report and Template\Script folders of the installation directory). Melanie will therefore ask you to choose the appropriate XSL template each time you print a report or history. Look at which template works with your browser and delete the other one. In this way, the software automatically opens the remaining file and does not ask you to make a choice.

10.3.3 Gel and report identifiers

Melanie allocates a unique identifier (ID) to each project, match set, gel image, etc. This is useful to assure data consistency, allow reliable identification of objects across a computer network and enable database integration.

The IDs in Melanie are UUIDs (Universal Unique IDentifiers), which are 128-bit numbers that are guaranteed to be unique through combinations of hardware addresses, time stamps and random seeds. These IDs allow the Melanie objects to be uniquely recognized. The software can therefore detect, for example, if you delete a gel and replace it with another one with the same name.

CAUTION! You must be very careful when manipulating project, match set, gel and other Melanie files outside the software, in order not to corrupt the data.

10.4 Export to spot excision robots

Please note that in addition to exporting spots to an excision robot, it may be useful to save them as part of a spot set, or annotate them. This allows you to easily select them later on to add experimental data such as mass spectrometry information.

10.4.1 Bruker Proteineer SP spot picker

Melanie can export spot coordinates of selected spots directly to the Bruker Proteineer SP spot picker, by choosing ***File > Export > Spots to Picker > Bruker Proteineer SP***. For more details about this functionality, please see the documentation provided by Bruker Daltonics.

10.4.2 GE Healthcare Ettan spot picker

To use the Ettan Spot Picker, two adhesive markers should be placed on the gel before scanning. These markers are used for the calibration of the coordinates, that is, for determining the correspondence between the X and Y positions of the analyzed gel image and the coordinates of the actual gel located on the spot picker (Figure 10-2).

Once the gel has been digitized and analyzed with Melanie, the software can generate a pick list. This list contains the location, in pixels, of the center of each spot you wish to pick, as well as the pixel coordinates for the centers of the two reference markers.

To export a file with spot coordinates for use by the spot picker, you first have to open the image files and perform image analysis (spot detection is mandatory). You should then annotate the reference markers. If a reference marker is well detected during the automatic spot detection process (nice round spot perfectly centered on the marker), you can just add an annotation on the marker spot. The basis of such an annotation is displayed as a small square, and its coordinates correspond to the center of the spot. If a reference marker is not well detected (irregular shape or consisting of several spots), it is better to delete the existing spot(s) and create an annotation on the pixel that is in the center of the marker. This kind of pixel annotation has a crossed basis and its coordinates correspond to the pixel it is attached to. Note that the two options can be used in a single gel (one marker with a spot annotation and the other with a pixel annotation), as long as the annotations are centered on the markers.

To create a pick list:

1 Identify the two reference markers on your image.

2 Zoom the image to better see the left reference marker.

3 Check if the marker is detected as a nice, round spot. If it is not, select the spot(s) on the marker and delete by choosing ***Edit > Spots > Delete***.

4 Click on the ***Select*** tool.

5 Double-click on the marker spot, or if such a spot is not present, on the pixel that is in the middle of the marker.

6 Select the ***Comment*** category.

7 Enter **IR1** as the label text.

8 If an annotation attached to a pixel is not in the center of the reference marker, you can move it to the appropriate position. Do this by clicking on its basis (cross) and holding down the left mouse button while dragging the annotation to its new position.

9 Move your gel to see the second (right) reference marker.

10 Repeat the procedure, but enter the label text **IR2** this time.

11 Once the two markers have been annotated, select spots to pick (Figure 10-2).

12 Choose ***File > Export > Spots to Picker > GE Healthcare Ettan***.

13 For each gel, you will be asked to save a pick list in text or XML format (only the text file can be read by the Ettan Spot Picker).

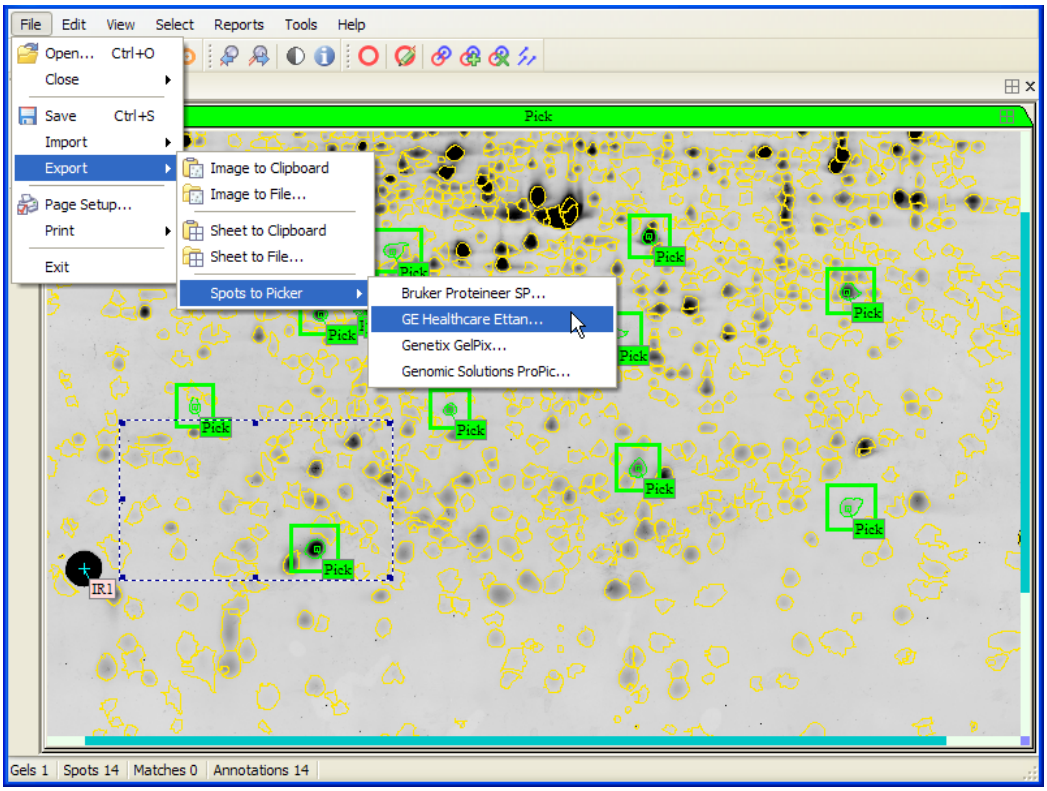


Figure 10-2. Reference markers IR1 and IR2. IR1 is attached to a pixel (cross basis). IR2 is attached to a spot. Both options can be used in a single gel. Spots to be picked are selected (highlighted in green).

10.4.3 Genetix GelPix spot picker

To export spot coordinates of selected spots directly to the Genetix GelPix spot picker, choose ***File > Export > Spots to Picker > Genetix GelPix***.

10.4.4 Genomic Solutions ProPic spot picker

The ProPic spot picker produces a TIFF file of the entire gel holder area with a resolution of 1035 x 1317 pixels, each pixel representing approximately 330 x 330 microns of the image area. This ProPic image can be analyzed with Melanie and spot coordinates in the image can

be exported back to the ProPic spot picker in a predetermined file format. The ProPic software subsequently translates the image X, Y coordinates into ProPic robot X, Y coordinates by using a robot map. Since the robot mapping assumes that each X, Y coordinate in the image always corresponds to the same position on the robot bed, the ProPic image must remain in its original form. It can never be resized, cropped or rotated.

The ProPic image is a picture of the current state of the gel from which the spots are to be picked. It is not necessarily the image used to determine the spots to pick. One can select spots from any analytical image (of the same gel) obtained from a different resolution system and analyzed with Melanie. However, the analytical image needs to be aligned to the ProPic image so that Melanie can export the aligned X and Y coordinates of the selected spots. Please bear in mind that proper alignment is crucial at this stage, especially for images of "old" gels that underwent significant shape change since they were originally imaged and analyzed, and for gel images that were acquired on a high-resolution system.

To export a spot coordinate file to the ProPic spot picker:

1 Inspect your analytical gel image in Melanie and select the spots you want to export to the spot picker. Annotate these spots or save them in a spot set so that you can easily re-select them at a later stage.

2 Place your gel on the ProPic robot bed, produce the ProPic image and open it in Melanie. Use the gel image as is. Do not resize, crop, flip, or rotate the image.

3 Align your analytical gel image to the ProPic image, using a sufficient number of landmarks.

4 Select all spots to be cut with the spot excision robot from the analytical image.

5 Make sure only the analytical gel image is selected.

6 Choose ***File > Export > Spots to Picker > Genomic Solutions ProPic***.

7 Enter a file name and destination folder. The file is automatically given a .tds extension (TwoDSpotlist).

8 Melanie exports the aligned X and Y coordinates for each selected spot. In other words, the coordinate system of the ProPic image will be used.

11 Undo, redo and history

11.1 Undo, redo

The software allows you to cancel any unwanted modifications to the images or layout of the current sheet by choosing ***Edit > Undo*** in the menu (Figure 11-1).

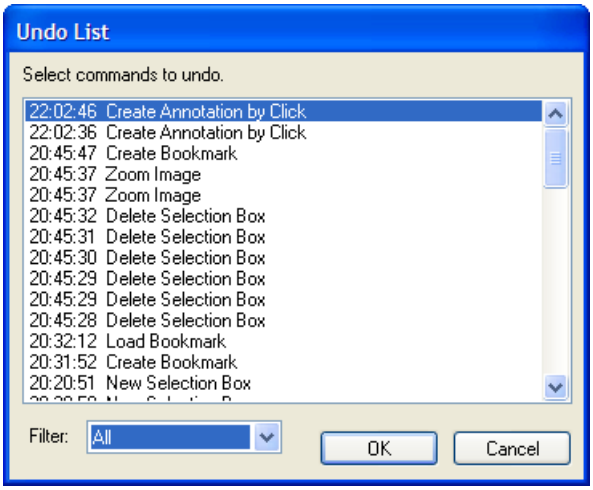


Figure 11-1. Undo window.

Each action in the Undo/Redo list is preceded by the time at which it was carried out. This information helps you to identify the particular sequence of actions that you would like to cancel.

By default, all actions are displayed. If you specifically want to track permanent changes that have been made to the image data (essentially the commands available under the Edit menu), then choose ***Only Edit*** from the ***Filter*** drop-down list.

All operations performed can be reversed except for modifications to the Workspace and functionalities linked to opening and saving files. This includes image rotation, flipping, cropping, and the inversion of gray levels.

To undo specific actions:

1 Choose ***Edit > Undo***.

2 Select a prior action to be undone.

3 Click ***OK***. The selected action and all following actions are undone automatically.

If you are not satisfied with your latest undo or you canceled too many operations, you can obviously reapply the actions.

To redo specific actions:

1 Choose ***Edit > Redo***.

2 Select the action to be redone.

3 Click ***OK***. The selected action and any preceding actions are redone.

11.2 History

You can display a history of the modifications that have been carried out during the present work session on the images or layout of the current sheet. Choose ***Edit > History > Show*** to open the History window (Figure 11-2).

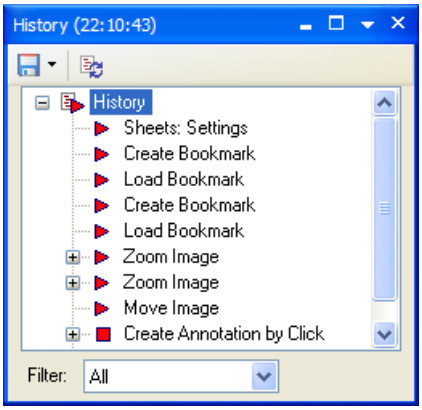


Figure 11-2. History window.

Please note that the action names displayed in the History window are the same as those used in Undo/Redo. As for Undo/Redo, you can choose ***Only Edit*** from the ***Filter*** drop-down list to restrict the displayed actions to those that bring about permanent modifications to the image data.

Some actions are preceded by a + or - node allowing the item to be expanded or collapsed. Once an action is expanded (by clicking on the plus sign), you see parameters and values that further describe the action.

Modifications to the Workspace and functionalities linked to opening and saving files (including image rotation, flipping, cropping, and inversion of gray levels) are not included in the History.

Insert marker

You can place a marker in the History by choosing ***Edit > History > Insert Marker***.

Clear

Clear the list of actions in the History by choosing ***Edit > History > Clear***.

Refresh

Please note that the content of the History is not automatically updated when you work on the images while the History window is still open. Click the ***Refresh*** icon to update the list.

Save, print, copy

By using the options in the ***Save*** icon drop-down list in the History window, the content of the History can be:

- Saved in an XML type file with the extension .hst.
- Printed. It will first display in your Web browser using the XSL stylesheet located in the Template\Script folder of the Melanie installation directory. Use the print option in your browser to get a paper copy.
- Copied to the clipboard.

Appendix A Shortcuts

A.1 Shortcut keys

Several menu commands can be activated by keyboard shortcuts. They are indicated to the right of the corresponding command in the menus. Please note the logic behind the key combinations:

Ctrl for gels.

Shift for spots.

Alt for annotations.

Ctrl + Shift for matches.

Some exceptions do exist. The most important ones are the following two shortcuts used for undoing and redoing actions carried out on a gel.

Shortcut	Menu Command
Ctrl+Z	***Edit > Undo...***
Ctrl +Shift+Z	***Edit > Redo...***

A.2 Tool shortcuts

Shortcut	Tool
Ctrl+1	Move
Ctrl+2	Zoom
Ctrl+3	Region
Ctrl+4	Selection
Ctrl+5	Measure
Ctrl+6	Landmark

A.3 Gel shortcuts

Shortcut	Menu Command
Ctrl+F	***View > Sheet > Navigation > Switch***
Ctrl+I	***View > Global > Grid Lines > Show***
Ctrl+J	***View > Global > Grid Lines > Edit...***
Ctrl+O	***File > Open...***
Ctrl+P	***View > Global > Show Profile***
Ctrl+S	***File > Save***
Ctrl+W	***File > Close > Sheet***
Ctrl+<Down>	***View > Gels > Navigation > Move > Down***
Ctrl+<Left>	***View > Gels > Navigation > Move > Left***
Ctrl+<Right>	***View > Gels > Navigation > Move > Right***
Ctrl+<Up>	***View > Gels > Navigation > Move > Up***
F2	***View > Show All***
F3	***View > Hide All***
F5	***Select > Unselect All***
Page Down	***View > Sheet > Navigation > Previous Page***
Page Up	***View > Sheet > Navigation > Next Page***
Shift+<Down>	***View > Gels > Navigation > Zoom > Out***
Shift+<Up>	***View > Gels > Navigation > Zoom > In***

A.4 Spot shortcuts

Shortcut	Menu Command
Shift+1	***View > Spots > Crossed***
Shift+2	***View > Spots > Outlined***
Shift+3	***View > Spots > None***
Shift+A	***Select > Spots > All***
Shift+E	***Edit > Spots > Edit Enabled***
Shift+N	***Select > Spots > Inverse Selection***
Shift+X	***Edit > Spots > Delete***

A.5 Annotation shortcuts

Shortcut	Menu Command
Alt+A	***Select > Annotations > All***
Alt+C	***Edit > Annotations > Labels > Copy***
Alt+D	***Edit > Annotations > Delete***
Alt+E	***Edit > Annotations > Labels > Edit...***
Alt+F	***Edit > Annotations > Add...***
Alt+H	***View > Annotations > Hide All***
Alt+J	***View > Annotations > Visible Categories...***
Alt+L	***Edit > Annotations > Link with Spot***
Alt+U	***Edit > Annotations > Unlink from Spot***
Alt+V	***Edit > Annotations > Labels > Paste***
Alt+X	***Edit > Annotations > Labels > Delete***
Alt+Y	***View > Annotations > Show All***

A.6 Match shortcuts

Shortcut	Menu Command
Ctrl+Shift+A	***Select > Matches > All***
Ctrl+Shift+G	***Edit > Matches > Add Match***

Shortcut	Menu Command
Ctrl+Shift+J	***View > Matches > Show ID***
Ctrl+Shift+K	***View > Matches > Hide All ID***
Ctrl+Shift+N	***Select > Matches > Inverse Selection***
Ctrl+Shift+U	***Edit > Matches > Delete Match***
Ctrl+Shift+Y	***View > Matches > Show Vectors***

Appendix B References

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B.3 Further reading

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